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Observations on the Metabolism of Tyrosine *O*[^{35}S]-Sulphate in the Rat

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Arylsulphate esters are normally present in appreciable amounts in mammalian urine where they appear as end products of the metabolism of phenolic compounds. They have not, however, with one notable exception, been shown to occur as normal constituents of mammalian tissues. The exception, tyrosine *O*-sulphate, is present in one of the two peptides which are released from bovine, rabbit and pig fibrinogens by the action of thrombin (Bettelheim, 1954; Blombäck, Boström & Vestermark, 1960; Blombäck & Sjöquist, 1960). The ester has also been detected in horse and human fibrin (Bettelheim-Jevons, 1958).

Several workers (see Dodgson, 1958) have shown that tyrosine *O*-sulphate cannot be synthesized from free tyrosine by the normal liver adenosine 3′-phosphate 5′-sulphatophosphate–phenolsulphokinase route. Moreover, the ester is not desulphated to any appreciable extent *in vitro* by the three arylsulphatases which are present in mammalian livers (Dodgson, Rose & Tudball, 1959). Attempts made by several workers (e.g. Grimes, 1959) to detect tyrosine *O*-sulphate in the urine of rats, rabbits, guinea pigs and mice have been unsuccessful, although Tallan, Bella, Stein & Moore (1955) noted that relatively large amounts of the ester were present in normal human urine.

The present work shows that tyrosine *O*-sulphate undergoes little or no desulphation when injected into rats, but is metabolized to yield two major products, one of which has been identified as the *O*-sulphate ester of *p*-hydroxyphenylacetic acid.

MATERIALS AND METHODS

Potassium tyrosine O[^{35}S]-*sulphate.* Material used in the quantitative experiments was prepared by the following modification of the method of Dodgson *et al.* (1959). Carrier-free $H_2{}^{35}SO_4$ (sp.gr. 1·84; 10·6 mc; 0·363 g.) was mixed with 2·3 ml. of unlabelled H_2SO_4 (sp.gr. 1·84) and the mixture cooled (with stirring) to $-7°$ in a refrigerated bath. L-Tyrosine (0·46 g.) was added in small portions over a period of 5 min., care being taken to avoid lumping. Stirring was continued at $-7°$ for a further 25 min., when the reaction mixture was poured into 100 ml. of crushed ice and water containing 20 g. of $Ba(OH)_2,8H_2O$. Precipitated $Ba^{35}SO_4$ was removed and excess of Ba^{2+} ions was precipitated by passing CO_2 through the solution at 0°. The precipitate was removed and the solution concentrated to dryness *in vacuo* at 38°. The residue was extracted with 30 ml. of water and the extract applied to a column (7 cm. × 1 cm.) of Dowex 50 ion-exchange resin (20–50 mesh; H^+ form) (Dow Chemical Co., Midland, Mich., U.S.A.). The column was eluted with 20 ml. of water and the eluate was adjusted to pH 7·0 with aq. 5% KOH before being concentrated to dryness *in vacuo* at 38°. The residue was dissolved in the smallest possible volume of cold water, and the potassium salt of tyrosine *O*[^{35}S]-sulphate precipitated by the addition of 12 ml. of ethanol and 16 ml. of ether. The material was kept overnight at 0°, separated at the pump, washed with ethanol followed by ether and dried *in vacuo* over $CaCl_2$ at room temperature (yield, 240 mg.). The radioactivity was approx. 0·7 μc/mg.

For the qualitative work material of greater specific activity was prepared by stirring unlabelled H_2SO_4 (0·605 ml.; sp.gr. 1·84) with 0·495 ml. of $H_2{}^{35}SO_4$ (sp.gr. 1·84; 41 mc) at $-5°$. L-Tyrosine (0·4 g.) was added over 15 min. The reaction tube was then broken into 50 ml. of crushed ice and water containing 7 g. of $Ba(OH)_2,8H_2O$ and the whole stirred vigorously. The subsequent procedure was as described above except for the smaller scale [10 ml. placed on a column (5 cm. × 1 cm.)]. The yield was 0·32 g. and the radioactivity approx. 6 μc/mg.

Descending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25, by vol.) (solvent *A*) showed both preparations to be homogeneous and free from inorganic [^{35}S]sulphate. Ultraviolet-absorption spectra and chromatographic mobilities agreed with those reported for unlabelled tyrosine *O*-sulphate (Dodgson *et al.* 1959).

p-Hydroxyphenylacetic acid O-sulphate. p-Hydroxyphenylacetic acid (1 g.; L. Light and Co. Ltd.) was added in small portions to 5 ml. of H_2SO_4 (sp.gr. 1·84) at $-5°$. After 30 min. the reaction mixture was poured into 200 ml. of crushed ice and water containing 35 g. of $Ba(OH)_2,8H_2O$. Precipitated $BaSO_4$ was removed by centrifuging and excess of Ba^{2+} ions was precipitated by passing CO_2 through the cold solution. After $BaCO_3$ was removed, the clear solution was dried *in vacuo* at 38° and the residue was extracted with 30 ml. of water. The extract was acidified (pH 3·0 with N-H_2SO_4) and extracted with four 40 ml. portions of ether to remove unchanged starting material, and the pH was adjusted to 8·0 with 5% KOH. Cold acetone (4 vol.) was added, and after 1 hr. precipitated K_2SO_4 was removed by filtration and the filtrate concentrated to dryness *in vacuo* at 38°. The material was dissolved in the minimum volume of cold water and 100 vol. of ether–ethanol (4:3) was added. The mixture was kept overnight at 0°, and the precipitated potassium *p*-hydroxyphenylacetic acid *O*-sulphate separated, washed with ethanol followed by ether and dried *in vacuo* over $CaCl_2$ at room temperature. Yield, 200 mg. (Found: ester $SO_4{}^{2-}$, 30·6; K, 12·6; loss at 100° *in vacuo*, 12·0. $C_8H_7O_6SK,2H_2O$ requires ester $SO_4{}^{2-}$, 31·4; K, 12·8; H_2O, 11·8%).

A ^{35}S-labelled sample of the compound was prepared as described above except that 0·3 g. of the free

phenol was sulphated with 1·5 ml. of $H_2{}^{35}SO_4$ (30·3 mc). The yield was 155 mg. and specific activity approx. 3·5 μc/mg.

Paper chromatography with solvent *A* and paper electrophoresis in 0·1 M-sodium acetate–acetic acid buffer, pH 4·5, showed both labelled and unlabelled samples to be homogeneous and free from inorganic sulphate.

Animals and collection of urine and faeces. M.R.C. hooded rats (2–4 months old) were fed, and urine and faeces collected, as described by Dodgson & Tudball (1960). When urine was collected for less than 6 hr. the animals were not given food or water during this period.

Measurement of radioactivity. The procedures described by Dodgson & Tudball (1960) were used.

Paper chromatography. Radioactive materials were located on chromatograms as described by Dodgson & Tudball (1960). The presence of unlabelled phenolic sulphate esters was demonstrated by spraying chromatograms with a 10% (v/v) solution of $HClO_4$ in ethanol and heating to 100° for 5 min. The dried paper was then sprayed with an aq. 0·5% (w/v) solution of diazotized sulphanilic acid before being exposed to NH_3 vapour. The free phenolic materials liberated as a result of the $HClO_4$ treatment showed as red-coloured spots.

EXPERIMENTAL AND RESULTS

Rats were injected intraperitoneally with tyrosine *O*[^{35}S]-sulphate dissolved in 0·5 ml. of water. Urine and faeces were collected after 24 and 48 hr. The animals were killed by a blow on the back of the head and the whole carcass was taken for the determination of ^{35}S. The results in Table 1 indicate that negligible amounts of the ester were desulphated *in vivo* and this is in accord with the results of the experiments *in vitro* of Dodgson *et al.* (1959).

The small amounts of inorganic [^{35}S]sulphate found in the urine could result from a biliary circulation of tyrosine *O*[^{35}S]-sulphate and subsequent desulphation of the ester by the intestinal flora. In order to check this possibility, three male rats were each given terramycin (total dose of 300 mg./rat) and sulphasuxidine (total dose of 1·5 g./rat) *per os* over a period of 70 hr. before injection. The bacterial content of faeces was examined before and after antibiotic treatment by a direct microscopic count of viable and non-viable cells and by counting viable organisms after 3-days growth on nutrient agar at 37°. The treatment resulted in a 100-fold reduction in the direct microscopic count and a 900-fold reduction in the number of viable organisms. Some decrease of the ^{35}S content of the inorganic sulphate fractions of the urines of these animals was obtained (see Table 1).

Paper chromatography of urines of rats injected with tyrosine O[^{35}S]-*sulphate*

Preliminary experiments showed that the bulk of the radioactivity of an injected dose (1·5 mg./200 g. body wt.) of tyrosine *O*[^{35}S]-sulphate

Table 1. *Distribution of* ^{35}S *in the urine, faeces and carcasses of rats injected intraperitoneally with tyrosine* O[^{35}S]-*sulphate*

Results are average values with the range in parentheses. ^{35}S was precipitated and counted as $Ba^{35}SO_4$ as described by Dodgson & Tudball (1960). The radioactivity of the various doses corresponded to approx. 4×10^3 counts/min./μmole, measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Sex	Dose (μmoles)	No. of animals	Range of body wt. (g.)	Distribution of ^{35}S in urine (% of ^{35}S injected)				Total ^{35}S content of faeces (% of ^{35}S injected)	Residual ^{35}S in carcass after 48 hr. (% of ^{35}S injected)
				Inorganic sulphate fraction		Combined inorganic and ester sulphate fraction			
				24 hr.	48 hr.	24 hr.	48 hr.		
Male	15·1	2	220	5·3 (4·6, 5·9)	1·0 (0·5, 1·5)	85·0 (83·0, 87·0)	2·0 (0·8, 3·2)	1·4 (0·6, 2·3)	3·9 (2·9, 4·9)
Female	15·1	2	180, 200	3·7 (3·0, 4·5)	—	87·6 (86·9, 88·3)	—	1·0*	2·1 (1·4, 2·7)
Male	3·7†	3	270–300	3·4 (3·2–3·7)	0·6 (0·4–0·9)	82·3 (74·6–89·9)	0·8 (0·7–1·0)	3·3*	1·5 (0·6–2·1)
Male‡	14·1	3	280–300	2·0 (1·7–2·2)	0·3 (0·1–0·5)	90·7 (78·0–100·0)	0·7 (0·5–0·9)	5·6 (2·5–8·8)	2·0*

* Results for a single animal only.
† Radioactivity corresponded to approx. 4×10^4 counts/min./μmole.
‡ Animals which have received terramycin and sulphasuxidine.

appeared in the urine within 6 hr. For the experiments described below, the 6 hr. urines from animals receiving the ester at this dose level were used. Sufficient urine was applied to Whatman no. 1 paper to give approx. 500 counts/min. (measured on the paper with a Panax monitor). Solvent system *A* and *tert.*-butyl alcohol–formic acid–water (8:3:4, by vol.) (solvent *B*) were used. Descending chromatograms of the urine were run overnight at room temperature. The following controls were run on the same papers as the test urines: (*a*) tyrosine *O*[^{35}S]-sulphate, (*b*) normal rat urine to which the ester had been added immediately before applying to the paper and (*c*) normal rat urine to which the ester had been added, the whole being kept at room temperature for 24 hr. before being applied to the paper. The chromatographic mobility of the ester in the presence of urine was slightly greater than that obtained in the absence of urine. Controls (*b*) and (*c*) showed a faint, slow-moving spot in addition to the main component. This faint spot, which was also present in the test urines, was assumed to be tyrosine *O*[^{35}S]-sulphate which had become bound to some urinary component, possibly protein or polysaccharide, since the intensity of the spot in (*b*) was the same as that of (*c*).

In some experiments, chromatograms of the urines of rats injected with tyrosine *O*[^{35}S]-sulphate showed the presence of small amounts of the unchanged ester. In other experiments no trace of the ester could be detected. In all chromatograms two main radioactive spots with R_F values approx. 0·28 and 0·5 in solvent *A* were detected. The corresponding value for tyrosine *O*[^{35}S]-sulphate in the presence of normal rat urine was 0·23. The relative intensities of the two main radioactive spots, as measured on the C100 Actigraph automatic strip scanner (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.), varied appreciably from experiment to experiment, and, in some cases, additional faint spots were observed on radioautographs.

Identification of one of the metabolites as p-*hydroxyphenylacetic acid* O[^{35}S]-*sulphate*

An unlabelled sample of the *O*-sulphate ester of *p*-hydroxyphenylacetic acid, when added to normal rat urine and chromatographed with *A*, had R_F 0·5. When the unlabelled ester was added to the test urines in amounts sufficiently great to be detected chemically on chromatograms, the red-coloured area obtained by the $HClO_4$–diazotized sulphanilic acid procedure coincided with the area of radioactivity at R_F 0·5, although some spreading did occur.

Paper chromatography. Two female rats were each injected intraperitoneally with tyrosine *O*[^{35}S]-sulphate (1·5 mg./200 g. body wt.) and the first urines voided were collected, pooled and chromatographed on several Whatman no. 1 papers with solvent *A*. A strip was cut from each paper and the fastest-moving metabolite was located by the automatic strip scanner. The corresponding areas were cut out from the remainder of each paper and eluted with water. The eluates were pooled and freeze-dried. The dried material was redissolved in water and rechromatographed on Whatman no. 1 paper with solvent systems *A* and *B*. Unlabelled *p*-hydroxyphenylacetic acid *O*-sulphate was run on the same papers as a marker. The chromatographic mobilities of the metabolite and the marker were identical.

Ultraviolet-absorption spectra. The freeze-dried material (from the urine of two rats) prepared as above was dissolved in water and applied to Whatman no. 1 paper as a single spot. Chromatography in solvent *A* was followed by elution of the appropriate radioactive area of the paper with water. As a control, an adjacent equal area of the paper was eluted with water. The test and control eluates were each freeze-dried and redissolved in 200 μl. of N-NaOH. The ultraviolet-absorption spectrum of the test solution was then measured in 1 cm. microcells with the Hilger Uvispek, the control solution being used in the blank cell. Maximum absorption was at 265 mμ (Fig. 1). An authentic sample of *p*-hydroxyphenylacetic acid *O*-sulphate also absorbed maximally at this wavelength in the presence of alkali (Fig. 1).

The test and control solutions were each mixed with an equal volume of 2N-HCl and heated to 100° for 15 min. Each was then extracted with 400 μl. of ether, the ethereal solution being subsequently re-extracted with 300 μl. of N-NaOH. The ultraviolet-absorption spectrum of the final alkaline test extract was determined as before, the alkaline control extract being used in the blank cell. Absorption maxima were obtained at 240 and 295 mμ (Fig. 1). An authentic sample of *p*-hydroxyphenylacetic acid had similar absorption maxima.

Isotope-dilution experiments. Three female rats were each injected with tyrosine *O*[^{35}S]-sulphate as above. Urine was collected for 5 hr., pooled and freeze-dried. The dried material was dissolved in 1 ml. of water and applied to a column (5 cm. × 1 cm.) of Dowex 50 ion-exchange resin (200–400 mesh, H^+ form). The column was washed with water at 0° and the acid eluate was collected at 0° in small fractions (2 drops). Two peaks of radioactivity were detected. The fractions corresponding to the first peak were shown, by paper chromatography, to contain the metabolite. These fractions were pooled, activated charcoal (20 mg.; British Drug Houses Ltd.) was added and the whole stirred intermittently at 0° for 1 hr. The charcoal was separated at the pump and washed with 1 ml. of cold water. The adsorbed radioactive material was eluted at room temperature with four 5 ml. portions of phenol-saturated water. Phenol was removed by extracting with four 5 ml. portions of ether and the residual aqueous solution was concentrated by freeze-drying. The concentrated solution was then chromatographed on Whatman no. 1 paper with solvent *A*. Radioactivity was located and the appropriate area of the paper eluted with water. The eluate was freeze-dried, dissolved in the smallest possible volume of water, applied to Whatman no. 1 paper and subjected to horizontal paper electrophoresis in 0·1 M-sodium acetate–acetic acid buffer, pH 4·6 for 5 hr. at a potential gradient of

14 v/cm. Radioactive material moved as a single zone, which was cut out and eluted with water. The eluate was freeze-dried and the dried material was dissolved in 0·5 ml. of an aqueous solution of 50 mg. of unlabelled *p*-hydroxyphenylacetic acid *O*-sulphate. Crystallization was achieved by the addition of ether–ethanol (4:3) drop by drop. The crystals were separated by centrifuging and dried *in vacuo* over $CaCl_2$ and P_2O_5 before being dissolved in 0·5 ml. of water and recrystallized as described above. After drying, a sample (3 mg.; sample 1) was retained and the remainder was recrystallized a further five times, a 3 mg. sample being retained after each recrystallization.

Each sample was dissolved in approx. 15 ml. of water in a precipitation tube (Dodgson & Tudball, 1960). 2·5 N-HCl (5 ml.) was added and the whole heated to 100° for 90 min. After cooling, 15 ml. of a 0·8% solution of carrier K_2SO_4 was added followed by 4 ml. of aq. 10% $BaCl_2$ (added drop by drop). The tubes were capped and kept without disturbance for 48 hr. The precipitated $BaSO_4$ was washed and plated (Dodgson & Tudball, 1960) and the radioactivity determined. Within the limits of experimental error, material of constant specific activity was obtained after the first recrystallization.

The material remaining after the final recrystallization was recrystallized and subjected to paper chromatography in solvent *A*. One component only was present on the chromatograms and the red-coloured area obtained by the $HClO_4$–diazotized sulphanilic acid spray coincided with the area of radioactivity.

Injection of p-*hydroxyphenylacetic acid* O[^{35}S]-*sulphate*

The second major metabolite (R_F 0·28) of tyrosine *O*[^{35}S]-sulphate could be an intermediate on the metabolic pathway between the ester and *p*-hydroxyphenylacetic acid *O*-sulphate or a product of the further metabolism of the latter compound. In order to check this second possibility, two female rats were each injected intraperitoneally with *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate (1·5 mg./200 g. body wt.). The urines were collected for 2·5 and 9 hr. and subjected to paper chromatography. Sufficient urine was applied to Whatman no. 1 paper to give approx. 500 counts/min. (Panax monitor) and solvent *A* and propan-1-ol–aq. 25% NH_3 soln.–water (6:3:1, by vol.) (solvent *C*) were used. Descending chromatograms were run overnight at room temperature and radioactive areas were located. Normal urine, to which *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate had been added, was run on the same papers as a marker. In both test and control urines some spreading of radioactive material occurred, but only a single radioactive zone was observed. Comparison of the chromatograms

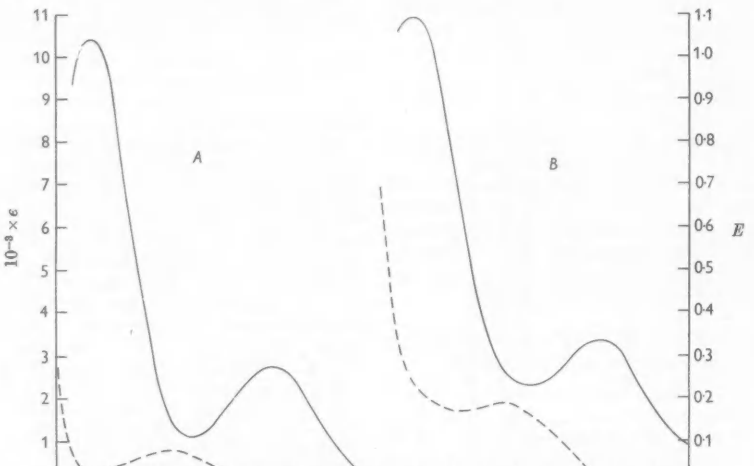

Fig. 1. Comparison of the ultraviolet-absorption spectra of *p*-hydroxyphenylacetic acid and the corresponding *O*-sulphate ester with those of the hydrolysed and unhydrolysed radioactive metabolite. For experimental details see text. *A*, Authentic samples of *p*-hydroxyphenylacetic acid (—) and *p*-hydroxyphenylacetic acid *O*-sulphate (- - -); the spectra are quantitatively related to each other but the scale of the *O*-sulphate ester has been increased five times. *B*, The radioactive metabolite after hydrolysis (—) and before hydrolysis (- - -); the curves are not quantitatively related to each other. All spectra were determined in the presence of N-NaOH.

of the 2·5 and 9 hr. urines showed that the bulk of the radioactivity appeared in the first period. No evidence of the presence, in the urine, of inorganic [^{35}S]sulphate or the second metabolite of tyrosine *O*-sulphate was obtained and it appeared that the injected material was being completely eliminated unchanged.

Two female rats were each injected with *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate as described above and the urines were collected for 5 hr. and pooled. One-half of the total volume of the urine was applied to a column (5 cm. × 1 cm.) of Dowex 50 ion-exchange resin (200–400 mesh, H^+ form) and the column was washed with water at 0°. The acid eluate was collected and the radioactive material adsorbed on charcoal, eluted and subjected to paper electrophoresis as described above. A single zone of radioactivity was obtained and eluted with water. The eluate was freeze-dried, redissolved in a small volume of water and subjected to paper chromatography with solvent systems *A*, *B* and *C*. The material was also subjected to paper electrophoresis. The radioactive material moved as a single spot in each solvent system and on paper electrophoresis. In each experiment the mobility of the spot was identical with that of authentic *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate used as a marker.

DISCUSSION

Quantitative experiments show that tyrosine *O*[^{35}S]-sulphate undergoes relatively little desulphation when injected into rats, and this is in accord with the finding (Dodgson *et al.* 1959) that mammalian arylsulphatases *A*, *B* and *C* show negligible activity *in vitro* against this arylsulphate ester. Further metabolism of the injected ester does occur, however, and often unchanged ester could not be detected in the urine. Of two principal metabolites formed, one has been identified as the *O*-sulphate of *p*-hydroxyphenylacetic acid. It has further been shown that this compound is eliminated unchanged when injected into rats, and it may therefore be the end product of tyrosine *O*-sulphate metabolism.

The principal route by which free L-tyrosine is degraded in the animal body is via *p*-hydroxyphenylpyruvic and homogentisic acids to fumaric and acetoacetic acids. The conversion of *p*-hydroxyphenylpyruvic acid into homogentisic acid involves the introduction of a second ring-hydroxyl group and subsequent rearrangement of the molecule so as to orientate the two ring hydroxyls in the 2- and 5-positions with respect to the side chain. It is usually accepted that the ring hydroxyl which was initially present in the *p*-hydroxyphenylpyruvic acid must be capable of a transitory existence in a keto form. Deamination of tyrosine *O*-sulphate to give *p*-hydroxyphenylpyruvic acid *O*-sulphate could not be followed by introduction of a second ring hydroxyl and subsequent intramolecular rearrangement, since the presence of the ester sulphate group would exclude the possibility of the existence of the initial ring hydroxyl in a keto form. Therefore it might be expected that the deamination of tyrosine *O*-sulphate would lead to an accumulation of *p*-hydroxyphenylpyruvic acid *O*-sulphate and this might, in turn, give rise (by aerobic decarboxylation) to the corresponding *p*-hydroxyphenylacetic acid derivative. Attempts to confirm the possibility that the unidentified metabolite which appears in rat urine after injection of tyrosine *O*[^{35}S]-sulphate is *p*-hydroxyphenylpyruvic acid *O*[^{35}S]-sulphate are being hindered by the difficulty of preparing an authentic sample of this ester.

Although it does not appear to have been established that tyrosine *O*-sulphate is a component of rat fibrinogen, the present work could well explain why previous workers (e.g. Grimes, 1959) have failed to detect the ester in rat urine.

SUMMARY

1. Tyrosine *O*[^{35}S]-sulphate, when injected intraperitoneally into rats, is metabolized without appreciable loss of ester sulphate.

2. Two major metabolites always appear in the urine. In some experiments small amounts of unchanged starting material can also be detected.

3. One of the major metabolites has been identified as *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate.

4. Injection of *p*-hydroxyphenylacetic acid *O*[^{35}S]-sulphate into rats is followed by the rapid elimination of the unchanged compound in the urine.

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Trialkyltins and Oxidative Phosphorylation

THE [^{32}P]PHOSPHATE–ADENOSINE TRIPHOSPHATE-EXCHANGE REACTION

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Both 2:4-dinitrophenol and trialkyltins influence the processes of oxidative phosphorylation in mitochondria. It is generally accepted that 2:4-dinitrophenol has a single action upon one of the steps of the energy-transferring chain between electron transport and the formation of adenosine triphosphate (for a full discussion and references see Aldridge & Parker, 1960; for definition of terms used to subdivide the processes of the respiratory chain, see Discussion). The stimulation of adenosine triphosphatase in mitochondria by 2:4-dinitrophenol is considered to be a consequence of the above-mentioned primary action. Trialkyltins inhibit both the oxygen uptake and adenosine triphosphatase stimulated by 2:4-dinitrophenol (Aldridge, 1958). Trialkyltins therefore act upon the energy-transferring chain which is involved in the action of 2:4-dinitrophenol (Aldridge, 1958; Aldridge & Parker, 1960).

The reversible exchange of orthophosphate with the phosphate of adenosine triphosphate is considered to be part of the energy-transferring chain in oxidative phosphorylation. This reaction has been demonstrated in mitochondria (Boyer, Falcone & Harrison, 1954; Boyer, Luchsinger & Falcone, 1956; Swanson, 1956; Löw, Siekevitz, Ernster & Lindberg, 1957) and in particles derived from mitochondria by various means but which still carry out oxidative phosphorylation (Cooper, Devlin & Lehninger, 1955; Cooper & Lehninger, 1957; Wadkins & Lehninger, 1957, 1959; Bronk & Kielley, 1957). 2:4-Dinitrophenol inhibits the exchange reaction at the same concentration as that which uncouples oxidative phosphorylation (Cooper & Lehninger, 1957; Boyer *et al.* 1956; Bronk & Kielley, 1958; Löw, Siekevitz, Ernster & Lindberg, 1958). In view of the association between the [^{32}P]phosphate–adenosine triphosphate-exchange reaction and oxidative phosphorylation and the inhibition by trialkyltins of some of the actions of 2:4-dinitrophenol, the influence of trialkyltins upon the exchange reaction has been examined.

METHODS

Abbreviations. The following abbreviations have been used throughout this paper: adenosine triphosphate (ATP) and adenosine diphosphate (ADP); radioactive phosphorus, ^{32}P; the exchange of ^{32}P (added as inorganic phosphate) with phosphorus atoms of ATP, ^{32}P–ATP-exchange reaction.

[^{32}P]*Phosphate-exchange reaction.* The medium used was basically that used for the assay of adenosine triphosphatase (Aldridge & Parker, 1960) but with an addition of inorganic phosphate to minimize the relative changes of inorganic phosphate concentration which might occur due to breakdown of ATP during the experiments. The rate of the exchange reaction was influenced by the phosphate concentrations, as shown in Fig. 1 (see also Cooper & Lehninger, 1957). For the standard test of ^{32}P exchange each beaker contained 3 ml. of a solution containing ATP (3 mM), KCl (10 mM), $MgCl_2$ (14 mM), ethylenediaminetetra-

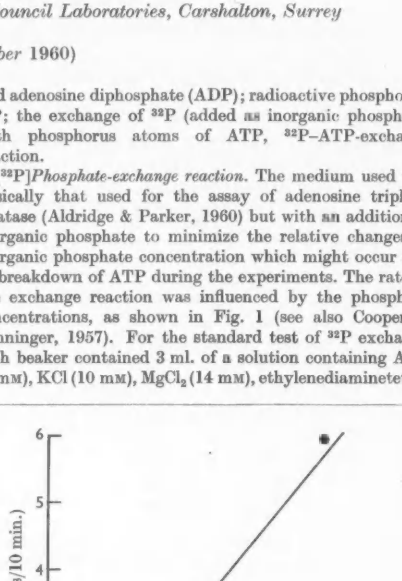

Fig. 1. Effect of phosphate concentration upon the ^{32}P–ATP exchange by mitochondria. Mitochondria (4·11 mg. of protein) were incubated for 10 min. at 37° in the medium used for the determination of adenosine triphosphatase (Aldridge & Parker, 1960) containing various amounts of inorganic phosphate. The concentration of phosphate plotted is the mean of the original and final determined concentrations. The liberation of phosphate during the incubation increased with increasing phosphate content of the medium and ranged from 0·4 to 1·8 μg.atoms of phosphorus. The μg.atom of phosphorus exchanged was calculated by the method of Boyer (1959).

acetic acid (1·0 mM), glycylglycine (16·7 mM), inorganic phosphate (3·3 mM), 1 μC of ^{32}P (equivalent to 200 000 recorded counts in the counting system used) and sucrose (30 mM). The beakers were shaken in air at 37°. After temperature equilibration 0·3 ml. of mitochondria suspension (equivalent to 150 mg. of original liver) was added and the mixture was incubated for 10 min. The reaction was stopped by the addition of 5 ml. of ice-cold 20% (w/v) perchloric acid. The precipitated protein was centrifuged off at 0° and the inorganic phosphate determined by the method of Fiske & Subbarow (1925). The concentrations and radioactivities of adenosine mono-, di- and triphosphates were determined as described by Stoner & Threlfall (1958) with 100 mg. of Norit charcoal and a sample of the perchloric acid extract of mitochondrial suspension equivalent to 60 mg. of original liver.

Under these conditions ATP and ADP were labelled with ^{32}P but the small amount of adenosine monophosphate contained a negligible amount of radioactivity. The results were calculated as counts/μg.atom of exchangeable phosphorus on the basis that the β- and γ-phosphorus atoms of ATP and the β-phosphorus atom of ADP were labelled. The incorporation of ^{32}P into the β-phosphorus atom of ADP is presumably due to myokinase present in rat-liver mitochondria (Barkulis & Lehninger, 1951). Particles derived from rat-liver mitochondria by treatment with digitonin do not contain myokinase activity and during the exchange reaction only the γ-phosphorus atom of ATP is labelled, whereas ADP is unlabelled (Cooper & Lehninger, 1957). In our experiments the activity of the β-phosphorus atom of ADP was always 60–80% of the mean activity of the β- and γ-phosphorus atoms of ATP.

Preparation of the mitochondria. Rat-liver mitochondria were prepared as previously described (Aldridge, 1957, 1958) with a Potter–Elvehjem-type homogenizer with a smooth glass tube and Perspex pestle with a total clearance of 0·02 in. The construction of the homogenizer has been described (Aldridge, Emery & Street, 1960).

Inhibitors. Triethyltin and tri-*n*-butyltin acetates were supplied by Dr G. J. M. Van der Kerk. These compounds were synthesized by published methods (Van der Kerk & Luijten, 1956). Dimethylformamide is an excellent solvent for trialkyltins, allowing stock solutions (usually 0·02 M) to be prepared. A sample (0·03 ml.) of these solutions was added to each beaker, final concentrations of 1% (v/v) dimethylformamide never being exceeded. This concentration has a negligible effect upon the activity of the control mitochondria.

Special chemicals. The following chemicals were obtained from the sources indicated: glycylglycine (Roche Products Ltd.), disodium salt of ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.) and radioactive phosphate (^{32}P, 1 mc/10 ml. in a solution of sterile iso-osmotic phosphate buffer, pH 7, containing 1 mg. of P/ml., The Radiochemical Centre, Amersham, Bucks.).

Analytical methods. Protein was measured by the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1957). Mitochondrial protein has been expressed as milligrams of albumin. Triethyltin and tri-*n*-butyltin were determined by the method of Aldridge & Cremer (1957) as modified by Cremer (1957).

Where sufficient determinations have been made the results are expressed as mean ± S.E. with the number of observations in parentheses.

RESULTS

Phosphate-exchange reaction

A range of concentrations of trialkyltins were used in the medium for the assay of the exchange reaction. The results given in Table 1 show that triethyltin prevents this reaction, a concentration of 0·4 μM causing 50% inhibition. Similar experiments with tri-*n*-butyltin (Table 2) showed that a concentration of 0·65 μM produced 50% inhibition.

Table 1. *Inhibition by triethyltin of the* [^{32}P]*phosphate–adenosine triphosphate exchange of liver mitochondria*

For details of techniques used see Methods section. Each set of figures represents a different preparation of mitochondria. The results are calculated upon the basis that ATP contains 2 exchangeable phosphorus atoms. 'Equilibrium' is the calculated specific activity at equilibrium of the inorganic and β- and γ-phosphorus atoms of ATP. On this basis the specific activity of the β-phosphorus atom of ADP was 60–80% of the mean activity of the β- and γ-phosphorus atoms of ATP. The concentration of triethyltin inhibiting the exchange by 50% (determined graphically) is 0·4 μM.

Concn. of triethyltin (μM)	Specific activity of ATP (counts/μmole of exchangeable P)			Mean (% of control)
	Expt. 1	Expt. 2	Expt. 3	
Nil	3080	2940	2850	100
2·5	—	320	477	14
1·25	678	555	695	22
0·625	—	895	1190	36
0·312	1880	1580	1825	63
0·156	—	2110	2270	76
0·078	2630	—	—	86
Equilibrium	7530	8000	6130	—

Table 2. *Inhibition by tri-n-butyltin of the* [^{32}P]*phosphate–adenosine triphosphate exchange of liver mitochondria*

For experimental conditions see Methods section and Table 1. The specific activity of the β-phosphorus atom of ADP was 80–90% of the mean activity of the β- and γ-phosphorus atoms of ATP. 'Equilibrium' is defined as in Table 1. The concentration of tri-*n*-butyltin inhibiting the exchange by 50% (determined graphically) is 0·65 μM.

Concn. of tri-*n*-butyltin (μM)	Specific activity of ATP (counts/μmole of exchangeable P)		Mean (% of control)
	Expt. 1	Expt. 2	
Nil	2580	3630	100
12·5	112	—	4
3·22	158	334	7
1·66	—	343	9
0·805	436	1050	23
0·415	—	2340	64
0·201	2050	3320	85
Equilibrium	6680	7350	—

In these experiments the medium contained 3–4 mM-inorganic phosphate and the gas phase was air. However, in a few preliminary experiments before the conditions were standardized, triethyltin and tri-*n*-butyltin were shown to inhibit the exchange reaction in the absence of added inorganic phosphate and with nitrogen as the gas phase.

Trialkyltins inhibit many other reactions associated with oxidative phosphorylation (Aldridge & Cremer, 1955; Aldridge, 1958). In Fig. 2 the effect of a range of concentrations of tri-*n*-butyltin upon six of these processes is shown. The results for each process are taken from a single experiment and in each experiment a different preparation of mitochondria was used. In Fig. 2 there is considerable scatter of the results around the line drawn through the mean values. In our experience this is the kind of reproducibility to expect from such experiments and particularly so with a steep inhibition curve. We therefore consider that the relationship between the concentration of tri-*n*-butyltin and the observed inhibition is identical for the different processes. The concentration of tri-*n*-butyltin inhibiting all of these processes by 50% falls within the range 0·6–1·0 μM and is for triethyltin between 0·2 and 0·4 μM.

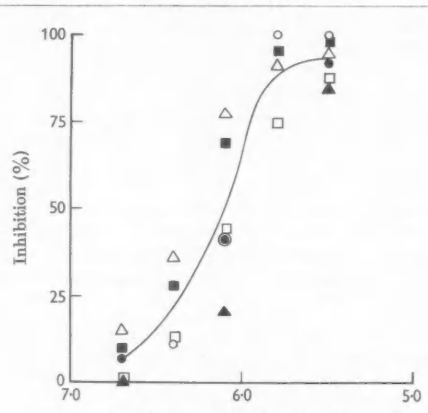

Fig. 2. Inhibition by tri-*n*-butyltin of various activities associated with the respiratory chain. With the exception of the ^{32}P–ATP exchange, the various activities were determined as previously described (Aldridge, 1958). Pyruvate+fumarate was the substrate in all the experiments involving O_2 uptake. ○, Adenosine triphosphatase activated by 2:4-dinitrophenol; ●, O_2 uptake induced by potato apyrase; △, ^{32}P–ATP-exchange reaction; ▲, O_2 uptake induced by 2:4-dinitrophenol; □, O_2 uptake in the presence of excess of hexokinase and glucose; ■, P uptake in the presence of excess of hexokinase and glucose. The line plotted passes through the means of the values obtained at each of the concentrations studied.

Distribution of triethyl- and tri-n-butyl-tin between mitochondria and aqueous medium

Oxidative phosphorylation can be carried out by particles derived from rat-liver mitochondria by treatment with digitonin (Cooper *et al.* 1955). A. L. Lehninger & C. L. Wadkins (unpublished observations; see Discussion) have shown that triethyltin and tri-*n*-butyltin inhibit oxidative phosphorylation carried out by these particles but the concentrations required were higher than those necessary with intact mitochondria. This suggested that intact mitochondria were able to concentrate these compounds and disrupted mitochondria were not. The results in Table 3 show that

Table 3. *Distribution of triethyltin between mitochondria and suspending medium*

Mitochondrial suspension (4 ml.) in 0·3 M-sucrose [equivalent to 2 g. wet wt. of liver and containing 51·8±2·1 (6) mg. of protein] was added to 4 ml. of medium containing triethyltin. The mixture was kept ice-cold for 5 min. and then centrifuged at 12 000*g* for 20 min. Triethyltin was determined in both the mitochondrial pellet and supernatant. The percentage recovery of added triethyltin was 95·6±1·9 (17). The distribution is the quotient [μg. of triethyltin/g. wet wt. of mitochondria divided by the concentration (μg./ml.) in the supernatant]. The wet wt. of mitochondria was calculated from the protein content and the results of Werkheiser & Bartley (1957).

Medium	Overall concn. of triethyltin (μM)	Distribution (mitochondria/medium)
0·3 M-Sucrose	40	67·9±4·9 (4)
Water	40	66 (1)
0·15 M-KCl	40	30·4±1·4 (8)
0·15 M-NaCl	40	31 (1)
Medium for adenosine-triphosphatase assay	40	24 (1)
Medium for ^{32}P–ATP-exchange reaction	17	25·3 (1)
	25	27·5 (1)
	33	25·2 (1)

Table 4. *Distribution of tri-n-butyltin between mitochondria and medium*

Conditions were as described in Table 3. Tri-*n*-butyltin could be determined in the supernatant [percentage recovery 99·0±1·8 (7)] but recovery from the mitochondrial pellet was never better than 50%. The solubility of tri-*n*-butyltin in 0·15 M-KCl at 0° is 0·36 mM.

Medium	Concn. of tri-*n*-butyltin added (μM)	Concn. of tri-*n*-butyltin in supernatant (μM)
0·15 M-KCl	14	0
	21	0
	29	1·1*
Medium for ^{32}P–ATP-exchange reaction	14	0
	21	1·1*
	29	2·0*

* These concentrations are near the limit of the method.

the concentration of triethyltin in mitochondria is 60–70 times that in the aqueous suspending medium when the medium is 0·3 M-sucrose and 25–30 times when the mitochondria are suspended in an ionic medium. Whether the cation is sodium or potassium does not appear to influence the distribution. Triethyltin could be readily determined in both the mitochondrial pellet and the suspending medium. Tri-*n*-butyltin could be determined in the supernatant but we were never able to recover more than 50% from the mitochondria. Determinations of tri-*n*-butyltin remaining in the suspending medium indicate that most of it is removed from the medium and presumably adsorbed on or concentrated in the mitochondria (Table 4).

DISCUSSION

For the purpose of this paper the following definitions have been used. The respiratory chain is regarded as a physiological entity concerned with all of the processes involved in the oxidation of substrates and the associated oxidative phosphorylation. The electron-transport chain is the structure in which occur the processes involving the transfer of electrons from coenzyme I through the flavin and cytochromes to oxygen. The energy-transferring chains are concerned with the processes whereby the energy of oxidation of electron-transport intermediates is used for the synthesis from inorganic phosphate of the terminal pyrophosphate bonds of ATP.

Triethyltin and tri-*n*-butyltin are potent inhibitors of the ^{32}P–ATP-exchange reaction in rat-liver mitochondria. When the respiratory carriers are maintained in a reduced state the ^{32}P–ATP exchange is inhibited (Wadkins & Lehninger, 1957; 1959) and it may be argued that since the trialkyltins prevent respiration this is the explanation of their effect upon the ^{32}P–ATP exchange. However, the conditions necessary to produce such inhibition must be carefully controlled [high concentrations of cyanide, or nitrogen as the gas phase, and an oxidizable substrate such as β-hydroxybutyrate must be present (Wadkins & Lehninger, 1957; 1959)]. It is clear, however, that without substrate and with intact mitochondria concentrations of respiratory inhibitors such as cyanide, antimycin A and amytal sufficient for complete inhibition of respiration produce less than 50% inhibition of the ^{32}P–ATP exchange (Jalling, Löw, Ernster & Lindberg, 1957; Löw *et al.* 1958; Boyer *et al.* 1956). The experiments with trialkyltins were carried out in the absence of added substrate and in air and there is close agreement between the inhibition of oxygen uptake and of the ^{32}P–ATP exchange (Fig. 2). The effect of trialkyltins upon the ^{32}P–ATP exchange cannot therefore be explained on the basis of an effect due to its inhibition of respiration.

The concentrations which are effective may be regarded as identical with those which inhibit other activities associated with the energy-transferring processes of the respiratory chain (Fig. 2). The inhibitory action of the trialkyltins upon the exchange reaction is in agreement with the widely held view that this reaction is associated with oxidative phosphorylation (Cooper & Lehninger, 1957; Boyer *et al.* 1956; Löw *et al.* 1958). The slope of the curve relating inhibition of the ^{32}P–ATP exchange to the concentration of tri-*n*-butyltin is very similar to and is probably identical with that for other processes associated with oxidative phosphorylation. The concentration of tri-*n*-butyltin (3·0 μM) producing almost complete inhibition of the exchange reaction also produces almost identical inhibition of the other processes. Therefore all of the ^{32}P–ATP exchange in rat-liver mitochondria is associated with the processes of oxidative phosphorylation.

A. L. Lehninger & C. L. Wadkins (unpublished observations) have shown that triethyltin and tri-*n*-butyltin affect oxidative phosphorylation and the ^{32}P–ATP-exchange reaction in particles derived from mitochondria by treatment with digitonin (Devlin & Lehninger, 1958). Both activities were inhibited by 1–10 μM-triethyltin and by 0·01–0·1 mM-tri-*n*-butyltin. The ADP–ATP exchange was not inhibited by these concentrations. The evidence for the association of the latter reaction with oxidative phosphorylation is not so strong as for the ^{32}P–ATP exchange. However, it is difficult to visualize a mechanism of ^{32}P–ATP exchange that would not involve an ADP–ATP exchange also. In addition the sensitivity of the ADP–ATP exchange in fresh preparations of submitochondrial particles to 2:4-dinitrophenol, dicoumarol and gramicidin is strong evidence for its association with oxidative phosphorylation (Wadkins & Lehninger, 1958). Trialkyltins do not inhibit the ADP–ATP exchange and in this respect they resemble azide (Wadkins & Lehninger, 1958; Lehninger, Wadkins & Remmart, 1959).

Trialkyltins inhibit the activities of intact and disrupted mitochondria associated with oxidative phosphorylation. This indicates that the whole structure of mitochondria is not required for the action of trialkyltins. However, the concentrations of trialkyltins required to inhibit oxidative phosphorylation in submitochondrial particles are higher than those for intact mitochondria. This difference is probably explained by the higher concentration of triethyltin and tri-*n*-butyltin found in intact mitochondria than in the surrounding medium. Intact mitochondria therefore concentrate trialkyltins and it is possible that the sub-

mitochondrial particles do not possess this property. This distribution of trialkyltins between intact mitochondria and the medium may modify our views on the intrinsic activity of each trialkyltin against the oxidative-phosphorylation system of mitochondria. The physical properties, and particularly solubility in lipid, vary appreciably in the different homologues and it is probable that each homologue will be differently distributed. In fact a further examination of the relation between their solubility in lipid and their concentration by intact mitochondria may provide evidence whether it is trialkyltin adsorbed upon the lipoprotein structure of the mitochondria which is active against oxidative phosphorylation.

2:4-Dinitrophenol stimulates adenosine triphosphatase in mitochondria and trialkyltins prevent this stimulation. Both 2:4-dinitrophenol (Cooper & Lehninger, 1957; Boyer *et al.* 1956; Bronk & Kielley, 1958; Löw *et al.* 1958) and trialkyltins inhibit the ^{32}P–ATP-exchange reaction. We considered that these relationships might enable us to decide whether or not 2:4-dinitrophenol was acting upon a phosphorylated intermediate. Schemes similar to that of Fig. 3 have been used by many workers in this field (Lehninger, 1953; Slater, 1953; Chance & Williams, 1956; Lardy, 1955). Slater's scheme (Slater, 1953; Myers & Slater, 1957) has been regarded as representative but an extra intermediate (Y) has been added to take account of the following considerations.

(*a*) Since the respiratory carriers associated with the three steps in electron transport associated with ATP formation are very different chemically it is unlikely that they will react with the same intermediate (I) and therefore three different intermediates will be involved.

(1)
$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2$$

(2)
$$A \sim I + X \rightleftharpoons X \sim I + A$$

(3)
$$X \sim I + Y \rightleftharpoons X \sim Y + I$$

(4)
$$X \sim Y + P_i \rightleftharpoons Y \sim P + X$$

(5)
$$Y \sim P + ADP \rightleftharpoons ATP + Y$$

Fig. 3. Hypothetical scheme for the energy-transferring chain in oxidative phosphorylation. The scheme is basically that of Slater (Slater, 1953; Myers & Slater, 1957), with an additional hypothetical intermediate Y to take account of views (Aldridge & Parker, 1960) that 2:4-dinitrophenol is acting upon an intermediate common to all of the three energy-transferring chains associated with electron transport (see Discussion). A and B represent coenzymes or respiratory pigments of the electron-transport chain.

(*b*) The three energy-transferring chains associated with electron transport have a similar sensitivity to 2:4-dinitrophenol and are therefore considered to consist of identical reactions between the point of action of 2:4-dinitrophenol and the reaction forming ATP (Aldridge & Parker, 1960).

(*c*) The ADP–ATP exchange is not itself sensitive to 2:4-dinitrophenol (Wadkins & Lehninger, 1958).

This scheme can now be used for discussion of the mode of action of 2:4-dinitrophenol and the trialkyltins. If it is assumed that all the reactions (1–5) are mediated by different enzymes (indicated by the arrows) then trialkyltins could exert their effects in two ways: by reacting either with an intermediate or with an enzyme. If an enzyme is inhibited, then, since trialkyltins inhibit at the same concentration both uptake of oxygen and adenosine triphosphatase stimulated by 2:4-dinitrophenol, the same enzyme must be involved in the action of these two substances (Aldridge, 1958). This enzyme cannot be that for reaction 5, the ADP–ATP exchange, since it is insensitive to trialkyltins. Since trialkyltins inhibit at the same concentration the adenosine triphosphatase stimulated by 2:4-dinitrophenol and the ^{32}P–ATP exchange, it seems that 2:4-dinitrophenol is reacting at some stage after the incorporation of phosphate (even if this be an enzyme complex involving phosphorus).

If trialkyltins are reacting with an intermediate to form an undissociated complex it would have to be with X to take account of the following. (*a*) As previously pointed out it is likely that three different intermediates (I_1, I_2, I_3) would be involved in the three energy-transferring chains associated with electron transport. Trialkyltins inhibit at least two of these chains (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Parker, 1960) and it is assumed that trialkyltins will react with an intermediate common to all three steps (therefore not I). (*b*) The ADP–ATP exchange is not affected by trialkyltin, so eliminating Y.

If trialkyltins react with X to form an undissociated R_2Sn–X, then reaction (2) will be prevented in the forward direction and reaction (4) in the reverse direction. 2:4-Dinitrophenol can therefore react only with X $\sim$ Y; reaction with Y $\sim$ P is excluded by the fact that the reaction (5), the ADP–ATP exchange, is not inhibited by trialkyltins. It is apparent from the same scheme that it is not possible to conclude whether 2:4-dinitrophenol reacts with a phosphorylated or a non-phosphorus-containing intermediate. Experiments have been carried out (Borst & Slater, 1959; 1960) to examine the requirement for phosphate of the stimulation by 2:4-dinitrophenol of the uptake of oxygen of rat-liver mitochondria with various substrates. It

was concluded that phosphate was not required for this stimulation and that 2:4-dinitrophenol reacts with an energy-rich intermediate in oxidative phosphorylation formed before the intervention of inorganic phosphate (Borst & Slater, 1960). The details of the relevant experiments have not yet been published but the report does not seem to eliminate the possibility that a small amount of inorganic phosphate was within the mitochondria and was 'turning over' rapidly. We therefore consider that the question of the involvement of phosphorus in the mechanism of action of 2:4-dinitrophenol is still not conclusively answered.

SUMMARY

1. Triethyltin and tri-*n*-butyltin inhibit the [^{32}P]phosphate–adenosine triphosphate-exchange reaction in rat-liver mitochondria. The exchange reaction and the other reactions associated with oxidative phosphorylation are all inhibited at the same concentration.

2. After incubation of rat-liver mitochondria in a medium containing trialkyltin, the concentration of trialkyltin is much higher in the mitochondria than in the medium.

3. These findings are discussed in relation to the mechanism of action of trialkyltins and 2:4-dinitrophenol upon the processes of oxidative phosphorylation.

We are very grateful to Professor A. L. Lehninger and Dr C. L. Wadkins for permission to mention their unpublished experiments and also to Professor A. L. Lehninger, Professor E. C. Slater and Dr P. Borst for much helpful discussion. We are also indebted to Miss M. O'Donnell and Mr B. W. Street for skilled technical assistance.

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Observations on the Binding of Haemoglobin and Haematin by Serum Proteins in the Rabbit, Rat and Guinea Pig

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When haemoglobin is added to human plasma, it is bound through the globin portion of the molecule to an α_2-globulin (Jayle, Boussier & Badin, 1952; Nyman, 1959). It has also been suggested that in human sera haemoglobin combines with albumin (Neale, Aber & Northam, 1958) and with a β_1-globulin (Neale *et al.* 1958; Tombs, 1960), but these complexes could not be found by Allison & ap Rees (1957), Aber & Rowe (1960) or Nyman (1960). Liang (1957) investigated the haemoglobin-binding properties of various animal plasmas and claimed that haemoglobin was bound by an α_2-globulin in the horse, dog, pig and duck, by albumin in the turtle, by fibrinogen in the ox, and by both an α_2-globulin and albumin in the frog.

In humans, the reaction between alkaline haematin and plasma proteins differs from that between haemoglobin and plasma protein. Heilmeyer (1933) was the first to suggest that a reaction took place between alkaline haematin and plasma proteins; Fairley (1938, 1941) showed that the reaction was between haematin and serum albumin and he termed the complex methaemalbumin. On electrophoresis, methaemalbumin migrates at the same rate as uncombined albumin (Fišer-Herman & Davorin, 1953). Other complexes besides 'haematin–albumin' have been described, but there is not complete agreement as to their rate of migration on paper electrophoresis. Fišer-Herman & Davorin (1953) describe a haematin–protein complex migrating in the α_2-globulin region; on the other hand, Neale *et al.* (1958), Aber & Rowe (1960) and Nyman (1960) have all described a haematin–protein complex travelling in the β-globulin. Moreover, Aber & Rowe (1960), using the technique of free-solution electrophoresis, also found haematin–protein complexes migrating in the α_2- and γ-globulin regions.

The reaction between alkaline haematin and animal plasma proteins has also been investigated. Fairley (1941) found that a complex was formed between rabbit albumin and alkaline haematin, although this complex did not give the characteristic absorption spectrum of human and simian methaemalbumin. Fišer-Herman & Davorin (1953), using paper electrophoresis, found haematin–protein complexes migrating in the albumin and in the α_2-globulin region in monkey plasma (*Macacus rhesus*); in the cow and horse they found a complex migrating in the albumin region, in the pig a complex migrated in the α- and β-globulin region, and in the chicken there were complexes in the albumin and α-globulin region.

Further observations are presented here on the binding of both haemoglobin and alkaline haematin by plasma proteins obtained from the rabbit, rat and guinea pig. The investigations were made with the technique of electrophoresis on cellulose acetate paper.

METHODS

Electrophoresis. Electrophoresis was carried out on cellulose acetate paper supported horizontally in air, with a current of 0·4 mA/cm. width of paper. The buffers were (1) barbiturate (*I* 0·05, pH 8·6) made according to the directions of White, Beaven & Ellis (1956), and (2) sodium phosphate (*I* 0·15, pH 6·4).

Nomenclature of proteins. The protein bands revealed by paper electrophoresis have been named on the basis of comparison with those of human serum. In the rabbit, a band migrating at the same rate as the β_1-globulin band of human serum has also been termed the β_1-globulin band. In the rat and the guinea pig, there is a band migrating at the same rate as the β_2-globulin of human serum and this has been termed the β_1-globulin band in these animals. All bands between the albumin and β_1-globulin have been termed α-globulins; in the rabbit and rat there are two bands, termed α_1- and α_2-globulin, and in the guinea pig three bands, termed α_1-, α_2- and α_3-globulin.

Establishment of the combining capacities of protein. The method of Nyman (1959) was used. This consists in adding increasing amounts of haemoglobin to constant volumes of serum and determining by paper electrophoresis the concentration of haemoglobin which just saturates the haptoglobins. Haemoglobin was added to give concentrations rising by 10 mg./100 ml. The haemoglobin present in the original serum was also determined and added to the calculated haemoglobin concentration. The result was then expressed to the nearest 5 mg./100 ml. Haemoglobin was stained with dianisidine by the method of Owen, Silberman & Got (1958) and proteins were stained with Ponceau S.

Estimation of the rate of turnover of the haemoglobin–haptoglobin complex. A rabbit whose total haemoglobin-binding capacity was estimated to be 40 mg. was injected with 60 mg. of haemoglobin. Plasma samples were obtained at frequent intervals over the following 24 hr. and

estimates of the haemoglobin-binding capacity in the presence of excess of haemoglobin made as follows. Two 20 μl. volumes of serum were placed side by side on Whatman no. 1 paper, and electrophoresis was carried out with phosphate buffer. The site of the haptoglobin–haemoglobin complex was determined by staining one of the electrophoretograms, and the complex eluted from the other by the method of Dent (1947) with 2 ml. of phosphate buffer. The haemoglobin content of the complex was estimated with dianisidine: to the eluate was added 1 ml. of acetic acid, 1 ml. of 0·1% (w/v) dianisidine in 10% (v/v) acetic acid, and 1 ml. of H_2O_2 (10 vol.). The extinction was determined at 460 mμ after 30 min. A solution of known haemoglobin concentration was used as a standard. The method gave a linear relationship between haemoglobin concentration and extinction and was not affected by the presence of phosphate or barbiturate. The factor normally present in serum which inhibits the colour development was not present in the eluates. The total plasma haemoglobin was also determined, with benzidine, and the free haemoglobin content calculated from the difference between the total and the combined haemoglobin.

Alkaline haematin was prepared by the method of Gatterman (1941).

RESULTS

Rabbit. A haemoglobin–haptoglobin complex migrated in the same position as the α_2-globulins (Fig. 1). No combination with albumin was detected with haemoglobin concentrations up to 400 mg./100 ml. The haemoglobin-combining capacities of the complex in the α_2-globulin region in seven adult rabbits (lop-eared variety) were 10, 15, 25, 25, 35, 45 and 45 mg. (average 30 mg.)/100 ml. of serum. Haematin in low concentrations formed a complex which migrated in the α_2-globulin region, and in higher concentrations a complex was also formed which migrated with albumin. In one experiment, haemoglobin saturated the complex in the α_2-region when the concentration reached 6 mM, whereas haematin saturated the complex at a concentration of 25 mM, that is, approximately four times the concentration required for saturation with haemoglobin. If just sufficient haemoglobin was added to serum to saturate the haptoglobin, then the further addition of haematin displaced the haemoglobin which appeared as free haemoglobin in the β-globulin region (Fig. 1).

The results of the experiment on the rate of turnover of the haemoglobin–haptoglobin complex in the rabbit are shown in Fig. 2. The initial half-time of removal of the complex was 40 min., and the free haemoglobin was also removed at approximately the same rate. Haptoglobin rapidly reappeared in the plasma and was raised above its original level 24 hr. later.

Rat. In sera obtained from Wistar rats, the haemoglobin–haptoglobin complex migrated with the α_2-globulins. Six estimates of the haemoglobin-combining capacity gave values of 25, 30, 30, 30, 30 and 50 mg./100 ml. of serum. On the addition of small quantities of haematin, a haematin–protein complex was found to migrate in the β_1-globulin region: at higher concentrations, a complex was also found migrating with albumin (Fig. 3). All the rat sera examined gave some staining with dianisidine in the α_2-globulin band, but

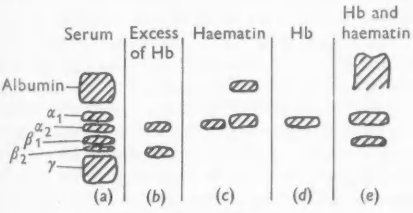

Fig. 1. Electrophoresis of rabbit sera in barbiturate buffer, pH 8·6. (*a*) Stained with Ponceau S; (*b*), (*c*), (*d*) and (*e*) stained with dianisidine. (*a*) Rabbit serum alone. (*b*) Rabbit serum with haemoglobin (final concentration, 100 mg./100 ml.). The free haemoglobin is in the β_2-globulin position. (*c*) Rabbit serum with haematin (final concentration, 1 mg. and 3 mg./100 ml.). (*d*) Rabbit serum with haemoglobin in a concentration insufficient to saturate the haptoglobin. (*e*) The haemoglobin–serum mixture used for (*d*) with the addition of haematin (3 mg./100 ml.). The haemoglobin which was attached to the α_2-globulin has been displaced by the haematin and is in the β_2-globulin position. Hb, Haemoglobin; α_1, α_2, β_1, β_2 and γ, corresponding globulins.

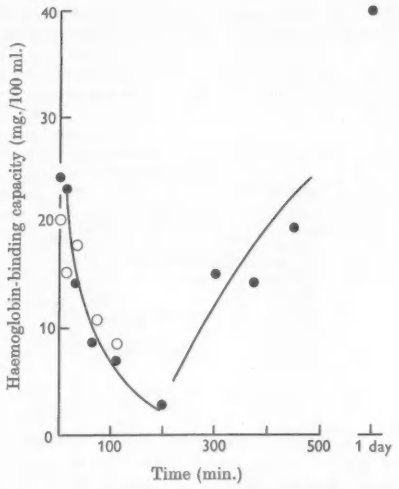

Fig. 2. Haptoglobin–haemoglobin (●) and free haemoglobin (○) in the serum of a rabbit after the injection of haemoglobin in excess of the haptoglobin-binding capacity.

since the addition of haematin did not appear to intensify the colour, it was considered that there was no haematin–protein complex migrating in the α_2-globulin region.

Guinea pig. When haemoglobin was added to guinea-pig serum, a haemoglobin–haptoglobin was first seen to migrate with albumin (Fig. 4). When the haemoglobin concentration was raised further, some of the sera showed a dianisidine-staining band in the β-globulin region. Although free haemoglobin also migrates in this region, this probably does not represent free haemoglobin as, on raising the haemoglobin concentration still further, another complex appears which migrates in the α_3-globulin region. When electrophoresis was carried out in phosphate buffer (pH 6·4) no staining appeared in the β-globulin region. It is possible that the complex migrating in the β-globulin region at pH 8·6 might migrate at pH 6·4 either with free haemoglobin or with the complex in the α_3-globulin region and thus be obscured. With phosphate buffer the binding capacities in the albumin region were 30, 50 and 50 mg./100 ml. of serum, and those of the complex travelling with the α_3-globulins was 20, 20 and 40 mg./100 ml. of serum in the three sera examined.

When haematin was first added to sera, a complex was formed which ran with albumin. Raising the concentration of haematin gave next a complex migrating in the β-globulin region; finally a complex appeared in the α_3-globulin region. In one serum a further complex was found in the α_1-globulin region.

When haemoglobin is added to human sera, haematin can be produced (Fairley & Bromfield, 1937). It is thus possible that the dianisidine-staining complexes seen after addition of haemoglobin to guinea-pig sera are really haematin–protein complexes. However, the complex migrating in the albumin region after addition of haemoglobin was slightly slower than the complex produced by the addition of haematin. Similarly, the haemoglobin complex in the α_3-globulin region ran slightly slower than the haematin complex. This suggests that haemoglobin and haematin form two distinct complexes. The complexes seen in the β-globulin region after addition of haemoglobin and haematin both ran at the same speed; there is thus the possibility that haemoglobin could be partially broken down to give a haematin–protein complex migrating in this region.

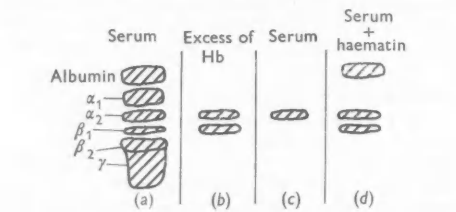

Fig. 3. Electrophoresis of rat serum in barbiturate buffer, pH 8·6. (*a*) Serum stained with Ponceau S. (*b*) Serum with the addition of haemoglobin (final concentration, 50 mg./100 ml.). (*c*) Serum alone. Some haemoglobin is present owing to haemolysis before separation of the serum. (*d*) The same serum as used in (*c*) with the addition of haematin (3·0 mg./100 ml.). (*b*), (*c*) and (*d*) were stained with dianisidine. For abbreviations see Fig. 1.

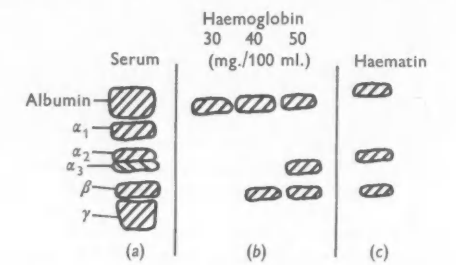

Fig. 4. Electrophoresis of guinea-pig serum in barbiturate buffer, pH 8·6. (*a*) Serum alone, stained with Ponceau S. (*b*) Serum with the addition of haemoglobin. (*c*) Serum with the addition of haematin (final concentration, 3 mg./100 ml.). (*b*) and (*c*) were stained with dianisidine. For abbreviations see Fig. 1.

DISCUSSION

The observations made by Fišer-Herman & Davorin (1953) and Liang (1957) have shown that there is considerable variation between animals and man in the ability of their plasma proteins to bind haemoglobin and haematin. The findings presented here supplement these observations. Of the sera obtained from the rabbit, rat and guinea pig, only that of the rat shows properties similar to human sera. As in human sera, the haemoglobin–haptoglobin complex of the rat migrates with the slow α-globulins only, and a haematin–protein complex migrates in the β-globulin and albumin regions. Aber & Rowe (1960) found in human sera that there was a tighter binding between the haematin and protein of the complex migrating in the β-globulin region than between the haematin and protein migrating in the albumin region; similarly, in the rat, the addition of haematin forms a complex in the β-globulin region before forming a complex in the albumin region, suggesting a tighter binding with the former than with the latter.

In the rabbit, a haematin–protein complex has been found which migrates with albumin. This supports the finding of Fairley (1941), who demonstrated a combination between albumin and

haematin by ultracentrifugal analysis. The haemoglobin–haptoglobin complex in the rabbit migrates in the α_2-region, as in humans. In humans the linkage in the haemoglobin–haptoglobin complex is between the globin and haptoglobin (Nyman, 1959) whereas in the rabbit there are two observations which suggest that the linkage may be through the haem portion of the molecule. First, the protein appears to bind four times as many haematin as haemoglobin molecules, suggesting that each binding site can take either four free haematin molecules or four haematins incorporated in a haemoglobin molecule. Secondly, the addition of haematin to a serum containing the haptoglobin–haemoglobin complex in the α_2-globulin region always resulted in the appearance of a dianisidine-staining compound in the position where free haemoglobin migrates, suggesting that free haematin displaces haemoglobin from the binding sites on the protein, and thus that the haemoglobin binds through haematin and not through globin. A further difference between human and rabbit haptoglobin is that rabbit haptoglobin is replaced within a few hours after its removal as haemoglobin–haptoglobin complex, whereas human haptoglobin takes several days to be replaced (Laurell & Nyman, 1957).

Guinea-pig sera differ from human sera in that the complex first formed on the addition of both haemoglobin and haematin migrates with the albumin. There is also the possibility that a haemoglobin–haptoglobin complex migrates in the β-globulin region, although, as discussed above, the dianisidine-staining material in this region could be a haematin–protein complex.

In connexion with the finding that haematin forms complexes migrating in the α-globulin region in the rabbit and guinea pig, Aber & Rowe (1960) found a complex migrating with the α-globulins and another with the γ-globulins in humans. This was only seen if the sera were examined by free electrophoresis; they could not be demonstrated on filter-paper electrophoresis, as the haematin was preferentially absorbed by the paper.

The results presented here on the binding of haemoglobin in the rabbit and rat do not agree with those of Liang (1957), who did not find any binding of haemoglobin by the proteins of plasma from rabbits and guinea pigs in Hong Kong. Murray & Connell (1960) also examined rabbit sera and found that they had an average binding capacity of only 10 mg./100 ml. of serum, which is distinctly lower than that of the rabbits examined in the present work. It is possible that there are considerable variations between different strains of rabbits and guinea pigs.

SUMMARY

1. The complexes formed by the addition of haemoglobin and haematin to sera obtained from the rabbit, rat and guinea pig have been investigated by paper electrophoresis.

2. In the rabbit, a haemoglobin–haptoglobin complex migrates with the α_2-globulins and a haematin–protein complex migrates with albumin and with the α_2-globulins.

3. In the rat, a haemoglobin–haptoglobin complex migrates with the α_2-globulins and a haematin–protein complex migrates with albumin and with the β_1-globulins.

4. In the guinea pig, haemoglobin–haptoglobin complexes migrate with albumin and α_3-globulin; in some sera, a complex probably also migrates with the β-globulins. Haematin–protein complexes migrate with albumin, α_3-globulin and β-globulin.

5. In the rabbit, the haemoglobin–haptoglobin complex was removed from the plasma with a half-time of 40 min.

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The Metabolism of Xylose by the Ocular Lens of the Rat

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Xylose and galactose differ markedly in their cataractogenic action. If young rats 28–32 days old are placed on a high galactose diet they develop dense lenticular opacity in 9–12 days, whereas rats 85–100 days old when placed on a similar diet require approximately 70 days for cataracts to develop (Mitchell & Dodge, 1935; Buschke, 1943; Babel, 1953; Lerman, 1960*b*). In contrast, when young rats are given a 30% xylose diet, although lens opacities do become manifest in 8–14 days, these lenticular changes are usually neither progressive nor permanent (with respect to lenticular transparency and function) in spite of the continued ingestion of xylose by the animal (Booth, Wilson & De Eds, 1953; van Heyningen, 1959). An apparent regression of the cataractous changes eventually occurs. Actually the lens (as well as the entire animal) is capable of recovering from, or adapting itself to, the initial deleterious effects of xylose. New fibres form around the original opacities and eventually the lens appears transparent. Furthermore, if older rats (14 weeks old) are placed on a prolonged 30% xylose diet, lenticular changes do not develop (Booth *et al.* 1953).

The sequence of events that occurs in the lenses of the young rats when these animals are fed on the 30% xylose diet was investigated in an attempt to elucidate some of the biochemical changes that occur in this organ during the period of the formation of lenticular opacities and the apparent regression of these changes.

MATERIALS AND METHODS

Holtzman-strain male white rats 28–32 days old, weighing approx. 45–55 g., were used in all the experiments. The experimental diet consisted of 30% of glucose, 30% of xylose, 21% of casein, 15% of fat, 4% of salt mixture W (Nutritional Biochemicals Corp., Cleveland, Ohio) and a complete vitamin supplement. Control animals received the same diet but with glucose replacing the xylose. Paired litter mates were used in these experiments; the animals were all started on their respective diets at the same time and were killed at specific time intervals as indicated in the Figures.

The relative activity of the hexose monophosphate shunt was determined on the basis of the recovery of $^{14}CO_2$ from the lenses incubated with glucose labelled in the C-1 or C-6 atom. The specific activity of this compound was 26000 counts/min./μmole of [1-^{14}C]glucose and 2700 counts/min./μmole of [6-^{14}C]glucose. The methods and materials employed in this experiment were reported by Lerman (1959*b*).

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed according to the method of Glock & McLean (1953).

Adenosine triphosphate (ATP) was determined by the firefly-luminescence method of Strehler & Totter (1952); disodium ATP (Sigma Chemical Co.) was employed as the standard.

Triphosphopyridine nucleotide (TPN)-xylitol dehydrogenase was assayed according to the method reported by van Heyningen (1959). Diphosphopyridine nucleotide (DPN)-xylitol dehydrogenase was assayed by a similar technique, in which DPN was substituted for TPN in the reaction mixture. D-Xylose (Mann Assayed Biochemicals, 136 Liberty St., New York) was used in these experiments. In the control reactions run concurrently with the TPN-xylitol-dehydrogenase assays, a significant amount of TPN was reduced although no xylitol had been added. It was thus necessary to determine the TPN-xylitol-dehydrogenase activity as the difference in the change in extinction at 340 mμ between the experimental and control reactions. This phenomenon, which occurred although the lens homogenates were dialysed for 16–18 hr. before assay, was never observed in the DPN-xylitol-dehydrogenase assays.

The oxidized and reduced di- and tri-phosphopyridine nucleotides DPN, TPN, DPNH and TPNH were determined by the method of Bassham, Birt, Hems & Loenig (1959), with slight modifications of the preliminary extraction procedure for the reduced pyridine nucleotides. Sodium carbonate (0·1 M) was used instead of NaOH (1 N) as greater stability and reproducibility were obtained. The standards employed (Sigma Chemical Co.) gave 80–85% recovery.

The soluble lens proteins were obtained by homogenizing single lenses in 1 ml. of water in micro-homogenizers kept in an ice–water bath. These tubes were then centrifuged at 3000 rev./min. for 30 min. and the supernatant fractions were retained for nitrogen determinations by the micro-Kjeldahl method.

RESULTS

The results of the experiment in which a single lens from each of eight control and 14 xylose-fed rats was incubated with [1-^{14}C]glucose are shown in Fig. 1. The mean recovery of $^{14}CO_2$ from eight control lenses was 864 counts/min./g. wet wt. of lens (range 568–1060 counts/min.; figures uncorrected for self-absorption, which averaged 80%). This value is in good agreement with the data

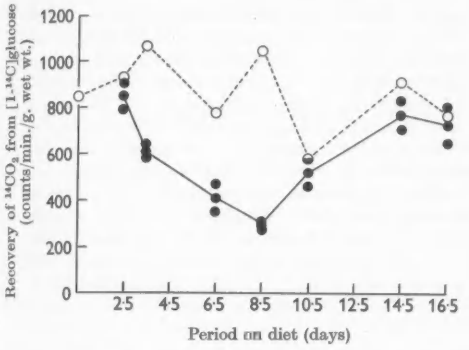

Fig. 1

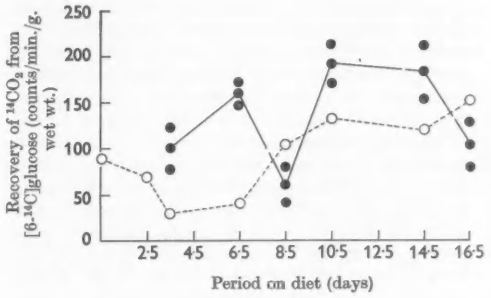

Fig. 2

Fig. 1. Recovery of $^{14}CO_2$ from the lenses of normal (○) and xylose-fed rats (●). Individual lenses were incubated for 21 hr. at 37° in 5 ml. of Krebs–Ringer bicarbonate buffer (pH 7·5) containing 50 μmoles of [1-^{14}C]glucose.

Fig. 2. Recovery of $^{14}CO_2$ from the lenses of normal (○) and xylose-fed rats (●). Individual lenses were incubated for 21 hr. at 37° in 5 ml. of Krebs–Ringer bicarbonate buffer (pH 7·5) containing 50 μmoles of [6-^{14}C]glucose.

reported previously for the normal rat lens (Lerman, 1959*b*). The recovery of $^{14}CO_2$ from the 14 lenses derived from the xylose-fed animals began to decline after the rats had been on the diet for 3 days. This decline became progressively more severe until the ninth day of the diet and then a slow progressive recovery took place and approached the control amounts by 15–16 days of the diet. These results correlate well with the time sequence of the development of lenticular opacities and the apparent recovery by this organ.

The amount of $^{14}CO_2$ recovered from the remaining lens of each of these rats (eight control and 14 xylose-fed) incubated with [6-^{14}C]glucose is shown in Fig. 2. The mean recovery for the control lenses was 92 counts/min./g. wet wt. (figures uncorrected for self-absorption, which averaged 80%); the corresponding values for the lenses derived from the xylose-fed animals, though somewhat higher on occasion, showed no statistically significant difference ($P > 0{\cdot}05$).

The results of the glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase assays are shown in Table 1. There was no significant variation in the activity of these two enzymes in the lenses from the xylose-fed rats as compared with the control lenses. These mean values (26·41 units of glucose 6-phosphate-dehydrogenase activity and 7·60 units of 6-phosphogluconate-dehydrogenase activity) for the control lenses are in close agreement with the data reported by Lerman (1960*a*).

Table 1. *Glucose 6-phosphate- and 6-phosphogluconate-dehydrogenase activity in the lens of the normal and xylose-fed rat*

For details see text. Enzyme activities are expressed as μg. of TPN reduced/mg. of soluble lens protein/hr.

Period on diet (days)	Glucose 6-phosphate-dehydrogenase activity		6-Phosphogluconate-dehydrogenase activity	
	Control lens	Lens from xylose-fed rat	Control lens	Lens from xylose-fed rat
3½	22·90	23·65	6·20	7·80
6½	31·90	32·90	8·30	8·10
9½	28·38	26·67	7·45	8·45
13½	26·05	27·67	8·85	6·85
16½	22·83	30·37	7·20	6·93

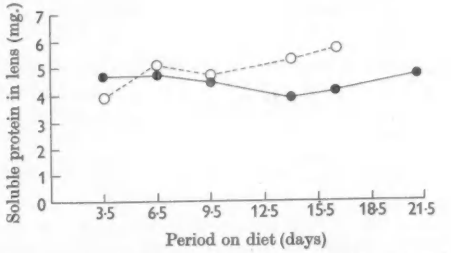

Fig. 3. Amount of soluble protein in lenses from normal (○) and xylose-fed rats (●) determined by the micro-Kjeldahl method.

The soluble-protein levels in the lenses derived from the xylose-fed rats remained relatively constant during the first 14 days of the diet, and then a small progressive increase in soluble-protein content per lens became evident, although by 21 days protein content in these lenses was still a good deal lower than the contents attained by the control lenses (Fig. 3).

Since the decreased level of soluble lens protein in the xylose-fed rats may be due to a lowered food intake (van Heyningen, 1959; Dische, 1959), the growth curves for these two groups of animals are shown in Fig. 4. After an initial failure to gain weight, the xylose-fed animals, after 6 days on the diet, showed an increase in body weight which paralleled that of the control animals.

The results of the ATP assays are shown in Table 2. In 14 control lenses the mean value was 38·41 μg. of ATP/lens (range 34·80–42·0). After 4 days on the xylose diet the amount of ATP per lens dropped by approx. 30% and remained relatively constant at this amount until 16 days of the diet, when it returned to the control values.

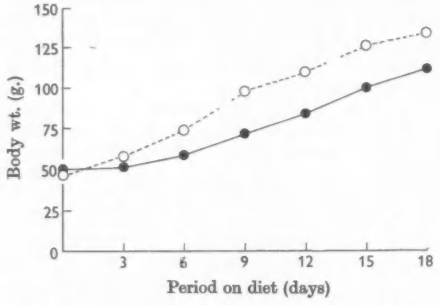

Fig. 4. Growth curves of paired litter-mate rats maintained on a control (○) and 30% xylose (●) diet.

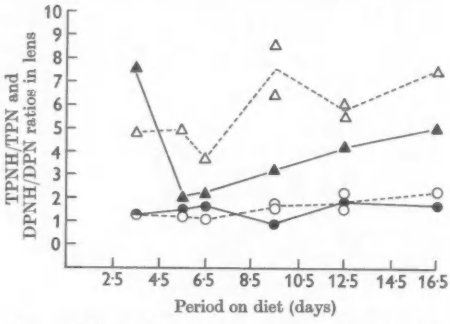

Fig. 5. TPNH/TPN ratio in the xylose-fed (▲) rat lens; TPNH/TPN ratio in the control (△) rat lens; DPNH/DPN ratio in the xylose-fed (●) rat lens; DPNH/DPN ratio in the control (○) rat lens. Each plotted point represents a single determination performed on two lenses.

The results of the determinations of the reduced and oxidized pyridine nucleotides are shown in Table 3 and Fig. 5. There was no change in the DPNH/DPN ratio in the lenses from the xylose-fed rats. However, the TPNH/TPN ratio showed a marked decline after 4 days on the diet and continued to fall until 6–8 days of the diet. A slow progressive recovery then ensued and the TPNH/TPN ratio returned to the control amount by the sixteenth day of the xylose diet. The marked fall in the TPNH/TPN ratio in these lenses was apparently due mainly to the decrease in total TPNH per lens. Once again this sequence of events is closely related to the clinically observed development and apparent regression of lenticular changes in these animals. Furthermore the high TPNH/TPN ratio in the control lenses provides added evidence about the high activity of the hexose monophosphate shunt in this organ.

TPN-xylitol (L-xylulose) dehydrogenase and DPN-xylitol (D-xylulose) dehydrogenase are both present in the lenses of the control and xylose-fed rats as previously reported by van Heyningen (1959). The activity of DPN-xylitol dehydrogenase is approximately ten times that of TPN-xylitol dehydrogenase (Tables 4 and 5). There is no change in the activity of the former enzyme in the lenses from the xylose-fed animals as compared with the control lenses. The activity of TPN-xylitol dehydrogenase in the xylose-fed rat lenses showed a marked fluctuation, but there was no

Table 2. *Concentration of adenosine triphosphate in the lens of the normal and xylose-fed rat*

ATP is expressed as μg./lens. Each value represents the average of two lenses.

Period on diet (days)	Control lens ATP	Control lens Wt. of lens (mg.)	Lens from xylose-fed rat ATP	Lens from xylose-fed rat Wt. of lens (mg.)
4	42·0	16·8	26	15·0
			27·5	19·0
			25·5	21·3
6	41·8	15·0	27·5	22·4
			29·0	22·5
			32·0	19·0
9	38·5	18·7	22·0	22·7
			26·8	28·5
			21·8	20·0
13	35·0	30·5	23·3	21·8
	34·8	25·9	23·3	27·0
			26·0	26·1
16	39·8	26·2	32·0	29·3
	37·0	30·5	35·5	27·8

significant difference between the activity of this enzyme when these values were compared with enzyme activity in the control lenses (Table 5).

DISCUSSION

The results of these experiments indicate that some of the biochemical difficulties that the lens of a xylose-fed animal encounters may be attributable to an impairment of the hexose monophosphate shunt. The decline in the C-1/C-6 and in the TPNH/TPN ratios after the third day on the diet and their subsequent return towards normal amounts (after the animal has been on the xylose diet for approximately 16 days) are related to the clinically observed changes that become manifest in the lens during this period. The development and apparent regression of the lenticular opacities occurs between 5 days and 14–18 days on this diet.

Since the activities of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase remain unimpaired, the mechanism whereby

Table 3. *Reduced and oxidized di- and tri-phosphopyridine nucleotides in the lens of the normal and xylose-fed rat*

Concentration of the nucleotides is expressed as μg./g. wet wt. of lens. Two lenses were employed for each determination.

Period on diet (days)	Control lens		Lens from xylose-fed rat		Control lens		Lens from xylose-fed rat	
	DPN	DPNH	DPN	DPNH	TPN	TPNH	TPN	TPNH
3½	141	180	124	169	51	247	29	221
5½	107	130	77	115	30	147	33	70
6½	191	206	103	170	36	131	35	79
9½	139	226	142	162	34	294	31	102
	160	273	—	—	31	201	—	—
12½	120	202	99	180	42	257	39	165
	118	273	—	—	38	211	—	—
16½	107	233	96	155	36	269	32	164

Table 4. *Diphosphopyridine nucleotide-xylitol-dehydrogenase activity in the lens of the normal and xylose-fed rat*

DPN-xylitol-dehydrogenase activity is expressed in units, 1 unit being defined as the activity which produces a change of E at 340 mμ of 0·001/min./g. wet wt. of lens at 24°.

Period on diet (days)	Wt. of control lens (mg.) (av. of two)	Enzyme activity in control lens	Wt. of xylose-fed lens (mg.) (av. of two)	Enzyme activity in xylose-fed lens
1	11·7	56·79	13·5	54·93
2½	18·6	59·14	12·0	98·61
3½	22·5	73·30	25·0	66·00
5½	19·4	73·79	21·8	72·63
6½	18·7	43·52	23·3	72·61
11½	23·2	37·35	24·8	45·03
		Mean 57·32		

Table 5. *Triphosphopyridine nucleotide-xylitol-dehydrogenase activity in the lens of the normal and xylose-fed rat*

TPN-xylitol-dehydrogenase activity is expressed in units, 1 unit being defined as the activity which produces a change of E at 340 mμ of 0·001/min./g. wet wt. of lens at 24°.

Period on diet (days)	Wt. of control lens (mg.) (av. of two)	Enzyme activity in control lens	Wt. of xylose-fed lens (mg.) (av. of two)	Enzyme activity in xylose-fed lens
1	11·7	5·12	13·5	3·70
2½	18·6	5·33	12·0	12·50
3½	22·5	2·96	25·0	2·67
5½	19·4	7·73	21·8	8·41
6½	18·7	7·84	23·3	1·43
11½	23·2	1·44	24·8	3·36
14½	19·2	5·65	26·8	7·46
		Mean 4·44		

the shunt is inhibited in these lenses differs from the previously reported effects of a high galactose diet on the rat lens (Lerman, 1960*a*; Lerman, 1959*a*). With the latter diet, a marked inhibition of the enzyme glucose 6-phosphate dehydrogenase rapidly develops. The mechanism responsible for an inhibition of the shunt in the lenses of xylose-fed rats is apparently a more complex one.

van Heyningen (1959) has reported the results of her studies on the metabolism of xylose by the rat lens. Her observations that the lactate concentration in the lenses of xylose-fed rats did not differ from the amount of lactic acid in the control lenses, together with our finding that the recovery of $^{14}CO_2$ from [6-^{14}C]glucose from both xylose-fed and control lenses was essentially the same, would indicate that there is no gross impairment of anaerobic glycolysis in the lens of the xylose-fed rat. We were also able to corroborate her report of the presence of DPN- and TPN-xylitol dehydrogenase in this organ. Although she did not find any significant difference in the concentration of ATP between the lens of the control and xylose-fed rat, her assay was done only at the third day of the diet. Our results indicate that there is a significant decrease in ATP concentration in these lenses, which occurs after 3–4 days of xylose feeding and returns to normal after 16 days in the diet. The apparent inhibition of soluble protein synthesis follows a more prolonged course and shows only slight signs of recovery by 14 days.

Since the evidence thus far points to a possible inhibition of the shunt followed by a decrease in ATP concentration and an apparent failure to synthesize soluble protein, the shunt may in some way be an important source for the formation of high-energy phosphates. Pirie, van Heyningen & Boag (1953) have reported the presence of TPNH–cytochrome *c* reductase in the rabbit lens. A relatively active TPNH– as well as DPNH–cytochrome *c* reductase is also present in the rat lens (S. Lerman, unpublished work). The activity of the TPNH–cytochrome *c* reductase was found to be approximately one-third greater than the DPNH–cytochrome *c* reductase. Furthermore, there is no change in the relative activities of these two enzymes in the lenses derived from xylose-fed rats (S. Lerman, unpublished work). Thus this mechanism may be capable of providing a source of ATP for the rapidly growing rat lens by utilizing the relatively large amounts of TPNH formed by the shunt.

In the lens of the xylose-fed rats, there is a marked fall in the TPNH/TPN ratio after 3–4 days on the diet, and recovery occurs by 16 days. The basis for this decline is the decrease in TPNH, since the amount of TPN remains fairly constant throughout this period. This loss may be due to the utilization of the TPNH by the rat lens in the reduction of D-xylose to xylitol (van Heyningen, 1959). This should become manifest quite early in the experimental period since the rat lens rapidly accumulates xylitol when the animal is placed on the xylose diet (van Heyningen, 1959). The equilibrium of this reaction apparently favours the formation of xylitol. van Heyningen has suggested that the limiting reaction might be the conversion of xylitol into D-xylulose. However, the activity of DPN-xylitol (D-xylulose) dehydrogenase remains unchanged in the lenses from the xylose-fed rats. It is thus possible that the rapid formation of and utilization of D-xylulose by the lens may also serve as a rate-limiting factor; that is, an inhibition of the hexose monophosphate shunt may develop.

SUMMARY

1. The lens of a 28- to 32-day-old rat fed on a 30% xylose diet shows an initial decline in the activity of the hexose monophosphate shunt as reflected by the C-1/C-6 ratio of $^{14}CO_2$ recovered from lenses incubated with [1-^{14}C]glucose or [6-^{14}C]glucose.

2. This C-1/C-6 ratio returns to normal amounts after the animal has been on the xylose diet for 15–16 days.

3. The TPNH/TPN ratio in these lenses shows a parallel decline and recovery whereas the DPNH/DPN ratio remains unimpaired.

4. Both DPN- and TPN-xylitol dehydrogenases are present in the lenses of control and xylose-fed rats, as previously reported by van Heyningen (1959). There is no significant alteration in their activities in the lenses from xylose-fed rats.

5. The amount of soluble protein in the lenses of xylose-fed rats suggests an inhibition of soluble protein synthesis in these organs after 6–8 days on the diet, with a slow recovery beginning at 14 days.

6. There is a moderate decline of adenosine triphosphate in these lenses after 4 days on the diet with a recovery occurring by 16 days.

7. An hypothesis has been proposed to explain the biochemical changes that occur in these lenses during the period of development and apparent regression of lenticular opacities.

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The Effect of SKF 525A (2-Diethylaminoethyl 2:2-Diphenylvalerate Hydrochloride) on Organophosphate Metabolism in Insects and Mammals

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The organophosphates are of two classes: those which are toxic directly, and those which require 'activation' in the animal body to produce the actual toxic compound. The directly acting compounds include the phosphates and phosphorothiolates, most of which contain the $-P(O)(OR)_2$ grouping, where R is an alkyl group. The other class includes the phosphorothionates, most of which contain the $-P(S)(OR)_2$ grouping, and the phosphorodiamidates that contain the $-P(O)(NR_2)$ grouping.

'Activation' is an oxidative reaction, involving the conversion in phosphorothionates of P(S) into P(O) (Gage, 1953) or, in phosphorodiamidates, of an alkyl into a hydroxyalkyl group (Heath, Lane & Park, 1955; Spencer, O'Brien & White, 1957). 'Activation' is accomplished in mammals almost exclusively by liver microsomes (Davison, 1955), and in insects principally by gut and fat body (Metcalf & March, 1953; O'Brien & Spencer, 1953).

It has been shown that, in mammalian-liver preparations, SKF 525A (2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride) blocks the 'activation' of schradan (octamethylpyrophosphoramide; tetramethylphosphorodiamidic anhydride), Guthion [*OO*-dimethyl *S*-(4-oxo-3H-1:2:3-benzotriazine-3-methyl) phosphorodithioate] and parathion (diethyl *p*-nitrophenyl phosphorothionate) (Davison, 1955; Murphy & DuBois, 1957). *In vivo*, SKF 525A is a potent antagonist of poisoning by schradan and Guthion, but not of poisoning by parathion (O'Brien & Davison, 1958). The present paper examines this paradox further.

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The effects of SKF 525A on the toxicity or metabolism of organophosphates in insects have not been reported previously. They are of interest because of the possibility that SKF 525A might be useful as an adjuvant in the treatment of livestock by systemic organophosphates for internal parasites. They may also help to elucidate the nature of the differences between the activating systems of mammals and insects.

METHODS

The mice were female albinos (20–35 g.) from Rolfsmeyer Farms (Madison, Wis.). The houseflies (*Musca domestica*) were 1-day-old adult females of a normal CSMA strain. The cockroaches (*Periplaneta americana*) were adult females, about 28 days moulted.

The organophosphates used were: (1) Chlorthion (dimethyl *m*-chloro-*p*-nitrophenyl phosphorothionate; (2) Co-ral (diethyl 3-chloro-4-methylcoumarin-7-yl phosphorothionate); (3) Delnav [mixed isomers of 2:3-*p*-dioxandithiol *SS*-bis-(*OO*-diethyl phosphorodithioate)]; (4) Diazinon (diethyl 6-methyl-2-*iso*propylpyrimidin-4-yl phosphorothionate); (5) dimefox (tetramethylphosphorodiamidic fluoride); (6) dimethoate [*OO*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate]; (7) Dowco 109 (*O*-*p*-*tert*.-butyl-*o*-chlorophenyl *O*-methyl *N*-methylphosphoramidothionate); (8) EPN (ethyl *p*-nitrophenyl phenylphosphonothionate); (9) mipafox (*NN'*-di*iso*propylphosphorodiamidic fluoride; (10) paraoxon (diethyl *p*-nitrophenyl phosphate); (11) parathion; (12) ronnel (dimethyl 2:4:5-trichlorophenyl phosphorothionate); (13) schradan; (14) Systox (diethyl 2-ethylthioethyl phosphorothionate and its thiolo isomer); (15) TEPP (tetraethyl pyrophosphate; diethyl phosphoric anhydride); (16) Tetram [*OO*-diethyl *S*-(2-diethylaminoethyl)phosphorothiolate]; (17) Thimet [*OO*-diethyl *S*-(2-ethylthioethyl)phosphorodithioate].

We are indebted to Chemagro Corp. for (1), (2) and (14), Hercules Powder Co. for (3), Geigy Chemical Co. for (4), Fison's Pest Control Ltd. for (5), American Cyanamid Co. for (6) and (11), Dow Chemical Co. for (7) and (12), E. I. du Pont de Nemours Co. for (8), Dr G. Schrader for (10), Monsanto Chemical Co. for (13), which was further purified by Dr E. Y. Spencer according to Casida, Allen & Stahmann (1954), Dr J. R. Robinson for (15), and Chipman Chemical Co. for (16). Compound (8) was purchased from L. Light and Co. The SKF 525A was presented by Smith, Kline and French Ltd. Lilly 18947 (2:4-dichloro-6-phenylphenoxyethyldiethylamine) was kindly given by Dr H. H. Moorefield. For the synthesis of radioactive parathion, 15 mc of $H_3{}^{32}PO_4$ in HCl was placed in a 16 mm. × 100 mm. Carius tube and the water and HCl were removed by a current of dry N_2. Then 355 mg. of $PSCl_3$ was added and the tube was sealed and heated 18 hr. at 400° in a muffle furnace (Vigne & Tabau, 1958). Subsequent synthesis of parathion was according to Krueger, O'Brien & Dauterman (1960). The yield was 71% based on $PSCl_3$, the activity 5700 counts/min. per μg./ml., in a liquid-counting Geiger tube.

Paraoxon (diethyl *p*-nitrophenyl phosphate) was prepared by passing N_2O_4 for 5 min. at room temperature through a solution in 10 ml. of methylene chloride of a second batch of ^{32}P-parathion prepared as above. The solvent was removed, the mixture taken up in benzene and the solution added to an alumina column (Woelm, acid, activity grade II). The excess of parathion (239 mg.) was washed through with 200 ml. of benzene, and the paraoxon then eluted off with chloroform. The yield was 20%, based on the original $PSCl_3$.

Toxicity determinations. The dose of SKF 525A used was the highest which, when injected alone, produced no symptoms. For mice, the SKF 525A (0·2% in 0·9% NaCl) was injected intraperitoneally at a dose of 20 mg./kg. Immediately after, the organophosphate in 0·2 ml. of propylene glycol/20 g. mouse was injected intraperitoneally. Mortalities were determined 24 hr. thereafter. For houseflies, the SKF 525A in 0·9% NaCl (1·3 μl./fly) was injected into the thoracic muscle, at 100 mg./kg. The organophosphates were dissolved in acetone and applied topically at 1·3 μl./fly to the thorax, immediately after the SKF 525A. With cockroaches, the SKF 525A in 0·9% NaCl (5 μl./g. wt.) was injected intra-abdominally at 300 mg./kg. The organophosphate, dissolved in the same volume of propylene glycol or ethanol–propylene glycol (1:9, v/v), was then injected intra-abdominally.

Parathion metabolism. Each mouse was injected intraperitoneally with [^{32}P]parathion (0·1% in propylene glycol) at a dose of 5 mg./kg., by using an Agla micrometer syringe. At the required time thereafter, the mouse was homogenized with 250 ml. of 5% trichloroacetic acid in a Waring Blendor for 2 min., then filtered through a wire screen. Three such preparations were pooled and counted, then 500 ml. was extracted with 100 ml. of benzene. The phases were separated by centrifuging and the benzene was dried with sodium sulphate and counted; 50 ml. of the benzene solution was passed through a 15 cm. × 1 cm. column of Woelm alumina grade II, and eluted with a further 50 ml. of benzene. Another 10 ml. of benzene was then passed through to check for complete elution, then 50 ml. of chloroform was passed through. The benzene fraction contained the parathion, and the chloroform contained the paraoxon (Plapp & Casida, 1958). Duplicate samples were counted in a liquid counter. In recovery experiments, 80% of injected parathion was recovered in the aqueous and benzene fractions; 96% of parathion added to a trichloroacetic acid-treated and filtered homogenate was extracted into the benzene.

Cockroaches were injected with [^{32}P]parathion (0·1% in propylene glycol) at 5 mg./kg. by means of an Agla syringe. Groups of five were homogenized in 250 ml. of 5% trichloroacetic acid, which was then extracted with 100 ml. of benzene. Subsequent fractionation was as described for the mouse.

Paraoxon metabolism. Mice were treated and homogenized as above, except that 1 mg. of [^{32}P]paraoxon/kg. was injected. A portion (500 ml.) of the pooled homogenates was extracted with 100 ml. of chloroform, the phases were separated by centrifuging and the chloroform phase (containing unchanged paraoxon) was dried with Na_2SO_4 and counted. In experiments in which [^{32}P]paraoxon was added directly to the homogenate before filtering, subsequent recovery into the chloroform of only 35% was obtained, although the recovery into the chloroform of paraoxon added to the filtered homogenate was 98%.

Cockroaches were injected and homogenized as described under 'Parathion metabolism', except that 1 mg. of [^{32}P]-paraoxon/kg. was injected. The 250 ml. of homogenate was extracted with 100 ml. of chloroform, which was dried with Na_2SO_4 and counted. In experiments in which [^{32}P]-paraoxon was added directly to the homogenate before filtering, subsequent recovery into the chloroform was complete (103%).

Schradan activation. One gut from a female cockroach was incubated in a 10 ml. beaker with 1·5 ml. of 0·05M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4, 0·25 ml. of 0·5% schradan and 0·25 ml. of water or SKF 525A solution. The beakers were shaken on the platform of a wrist-action shaker for 90 min. The solutions from four were pooled and duplicate 1 ml. samples added to a Warburg flask containing 1 ml. of a cholinesterase preparation (laked human erythrocytes at one-third their blood concentrations) and 0·5 ml. of 1·5% $NaHCO_3$. The standard Warburg assay was used at 25°, with 0·2 ml. of 2% acetylcholine bromide as substrate.

RESULTS

Studies on mammals and their tissues

Toxicity. Table 1 shows that in mice the only phosphorothionate against which SKF 525A provided substantial protection was dimethoate. Further work showed that the LD_{50} of dimethoate was raised from 80 to 195 mg./kg. by the SKF 525A. This 2·4-fold increase is comparable with the 1·6–2·0-fold increase previously reported for Guthion (O'Brien & Davison, 1958). It appears that protection against phosphorothionates is the exception rather than the rule. With Thimet a distinct synergism was noted. Previous work (O'Brien & Davison, 1958) had shown the SKF 525A did not protect mice from parathion poisoning. A brief study showed that neither did it synergize para-

thion poisoning, e.g. 10 mg. of parathion/kg. killed 10% of control mice or of mice treated with 20 mg. of SKF 525A/kg. (duplicate batches each of ten mice were used).

In a brief study on rats, involving 28 animals, little or no protective action against dimethoate was found, e.g. 350 mg. of dimethoate/kg. killed 89% without or 78% with SKF 525A.

Protection against all three phosphorodiamidic compounds was given by SKF 525A in mice. Further studies with mipafox indicated about a 1·6-fold increase in the LD_{50}; this may be compared with the fourfold increase reported for schradan (O'Brien & Davison, 1958). Table 1 indicates that for dimefox the increase in LD_{50} must be substantially less than twofold.

The compound Lilly 18947 is an inhibitor of barbiturate degradation (Fouts & Brodie, 1956). Since it has been shown that such compounds antagonize schradan poisoning (O'Brien & Davison, 1958), Lilly 18947 was tested in the mouse in the same way as SKF 525A, by using the hydrobromide salt. It was an effective antagonist; 60 mg./kg. protected fully against the standard 25 mg./kg. dose of schradan which alone gave 67% mortality. However, it was less effective than SKF 525A, weight for weight: at 20 mg. of antagonist/kg. the standard schradan dose gave 31% mortality with Lilly 18947, compared with 0% for SKF 525A.

With phosphates and phosphorothiolates, no activation is required; consequently if SKF 525A acts solely by blocking activation, it should have no effect in these cases. This was confirmed with TEPP and paraoxon (Table 1). Tetram was studied because of the report of Scaife & Campbell (1959) that the degradation of this compound was accomplished by liver microsomes and was inhibited *in vitro* by SKF 525A. If this was also true *in vivo*, SKF 525A should synergize Tetram poisoning. This was confirmed (Table 1). Further examinations showed that 20 mg. of SKF 525A/kg. lowered the normal LD_{50} of Tetram (0·55 mg./kg.) to 0·20 mg./kg.

Metabolism. The essential paradox to resolve was: why does SKF 525A antagonize parathion 'activation' by liver homogenates, yet have no saving effect on parathion poisoning? The first point examined was the effect of SKF 525A on [^{32}P]parathion metabolism in the intact mouse. Fig. 1 shows that the production of paraoxon (the actual toxic compound) was substantially increased by SKF 525A *in vivo*, in contrast with the results expected from the report on liver homogenates (Davison, 1955). There was negligible effect on the levels of parathion or degradation products.

A study was then made of the production by liver slices of paraoxon from parathion. Table 2 shows that paraoxon production was substantially inhibited by mM-SKF 525A, the concentration used by Davison. However, Gaudette & Brodie (1959) found that high concentrations (5 mM) of SKF 525A caused non-specific inhibition of microsomal reactions, owing to protein denaturation; at 0·4 mM, this non-specific effect disappeared. The

Table 1. *Effect of SKF 525A on the toxicity of organophosphates to mice*

The appropriate dose was first established approximately with groups of three mice, then duplicate groups of ten mice were used for each dose. If agreement to the nearest 10% was not obtained, further groups of ten were tested until three consecutive groups agreed within 10%. The SKF 525A dose was 20 mg./kg.

	Organophosphate dose (mg./kg.)	Percentage mortality alone	Percentage mortality with SKF 525A
Phosphorothionates			
Chlorthion	140	80	60
Co-ral	24	67	83
Dimethoate	90	70	0
Delnav	55	70	90
Diazinon	15	20	30
Dowco 109	600	90	70
EPN	15	40	50
Ronnel	800	50	50
Thimet	3	30	90
Phosphorodiamidic compounds			
Schradan	25	78	10
Dimefox	1	20	0
Dimefox	2	100	100
Mipafox	30	70	10
Phosphates and phosphorothiolate			
Paraoxon	2·5	60	60
TEPP	6	0	0
TEPP	7	100	100
Tetram	0·45	0	100

Table 2. *Effect of SKF 525A on oxidation of parathion to paraoxon* in vitro

Results are given as total μg. of paraoxon produced by five cockroach guts or 0·5 g. of sliced mouse liver. Each figure is an average from two experiments.

Tissue	Alone	With SKF 525A (mM)	Inhibition (%)	Alone	With SKF 525A (0·4 mM)	Inhibition (%)
Liver slices	4·17	1·28	69	5·33	3·90	27
Whole cockroach guts	6·68	1·93	71	4·93	3·36	32

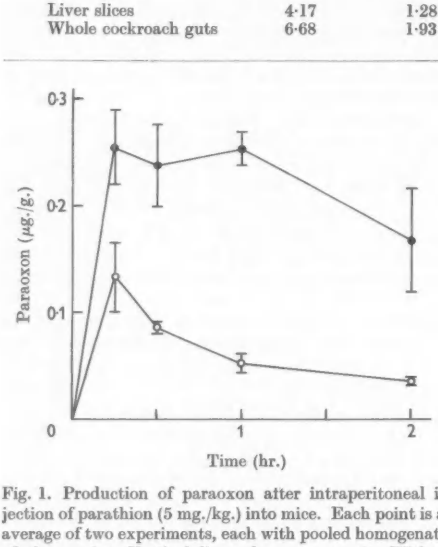

Fig. 1. Production of paraoxon after intraperitoneal injection of parathion (5 mg./kg.) into mice. Each point is an average of two experiments, each with pooled homogenates of three mice. Vertical lines show range. ○, Without SKF 525A; ●, with SKF 525A (20 mg./kg.).

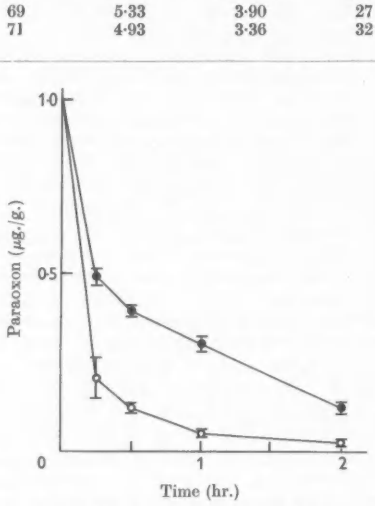

Fig. 2. Degradation of paraoxon after its intraperitoneal injection (at 1 mg./kg.) into mice. Details are as in Fig. 1. Results are corrected for partial recovery.

liver-slice work was therefore repeated with 0·4 mM-SKF 525A; as Table 2 shows, inhibition of paraoxon production was still found. Davison's results are therefore confirmed; but the results *in vivo* and *in vitro* are in conflict.

The only explanation that would resolve the conflict would be that paraoxon degradation *in vivo* was inhibited by SKF 525A. This rather improbable hypothesis was examined by injecting [^{32}P]paraoxon into mice and studying its degradation. As Fig. 2 shows, paraoxon degradation was indeed inhibited by 20 mg. of SKF 525A/kg.

Studies on insects and their tissues

Toxicity. As schradan is not toxic to the cockroach and housefly, it could not be tested in this study.

Against the cockroach, only Guthion, paraoxon, parathion and ronnel were tested. A clear-cut synergism, instead of the expected protection, was found with Guthion: the LD_{50} was lowered by SKF 525A treatment from 2·5 to 0·5 mg./kg. The plots of probit-of-mortality against organophosphate dose were not parallel for the two treatments (i.e. with and without SKF 525A), so that the LD_{85} was slightly increased by the SKF 525A, from 5·0 to 5·5 mg./kg. The toxicity of parathion was unaffected: the LD_{50} at 1 week was 0·9 mg./kg. with or without SKF 525A. However, the agent delayed the toxic effects; after one day, 1 mg. of parathion/kg. killed or prostrated an average of 80%, but with SKF 525A this figure was reduced to 10%. The toxicity of paraoxon was synergized: thus 0·5 mg. of paraoxon/kg. killed an average of 20%, but with SKF 525A this figure was increased to 80%. With ronnel, slight synergism was found: the LD_{50} was 59 mg./kg. without and 51 mg./kg. with SKF 525A.

The results with the fly are shown in Table 3. For Guthion and Diazinon distinct synergism was found; in the other four cases there was little effect.

Metabolism. The oxidation of [^{32}P]parathion to paraoxon was studied in the living cockroach. As Fig. 3 shows, although SKF 525A inhibited paraoxon formation to a small extent during the first 30 min., yet by 1 hr. it had almost doubled the paraoxon level. This second phase of the effect resembles that found in the mouse (Fig. 1).

Paraoxon production from cockroach gut *in vitro* was next studied, with the two SKF 525A concentrations which had been used for mouse liver. As Table 2 shows, SKF 525A substantially inhibited paraoxon production, to an extent remarkably similar to that found for liver.

Finally the degradation of injected [^{32}P]paraoxon was examined. Fig. 4 shows that paraoxon was degraded less rapidly in the cockroach than in the mouse, and that the degradation was inhibited by 20 mg. of SKF 525A/kg. As in the mouse, this finding accounts for the fact that SKF 525A causes increased concentrations of paraoxon after injection of parathion, in spite of its inhibitory effect on paraoxon synthesis.

In order to study the effects of SKF 525A upon 'activation' of schradan by cockroach guts, schradan was incubated with the guts and samples were taken after (usually) 60 min. for anticholinesterase assay of the 'activation' product. Fig. 5 shows for schradan that activation was roughly linear for 1·75 hr., and also demonstrates the considerable variation with individual guts. Consequently in subsequent work quadruplicate treatments were pooled and duplicate samples then taken for assay.

Table 3. *Effect of SKF 525A on the toxicity of phosphoro-thionates and -dithoates to houseflies*

The procedure was as in Table 1, except that groups of 20 houseflies were used.

Insecticide	LD_{50} alone (mg./kg.)	LD_{50} (mg./kg.) with 10 mg. of SKF 525A/kg.
Co-ral	1–2	1–2
Dimethoate	0·4–0·8	0·4
Diazinon	1	0·5
Dowco 109	Approx. 8	Approx. 8
Guthion	4	2
Ronnel	1	1

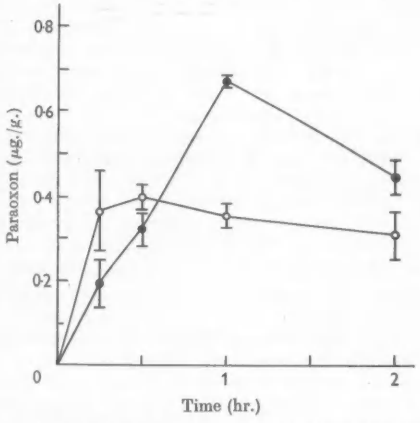

Fig. 3. Production of paraoxon after injection of parathion (5 mg./kg.) into cockroaches. Each point is an average of two experiments, each with a homogenate of five insects. Vertical lines show range. ○, Without SKF 525A; ●, with SKF 525A (20 mg./kg.).

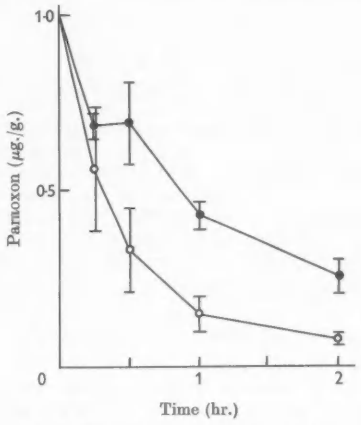

Fig. 4. Degradation of paraoxon after its injection (at 1 mg./kg.) into cockroaches. Each point is an average of three or five experiments, each with a homogenate of five insects. Vertical lines show standard deviation. Results are corrected for partial recovery. ○, Without SKF 525A; ●, with SKF 525A (20 mg./kg.).

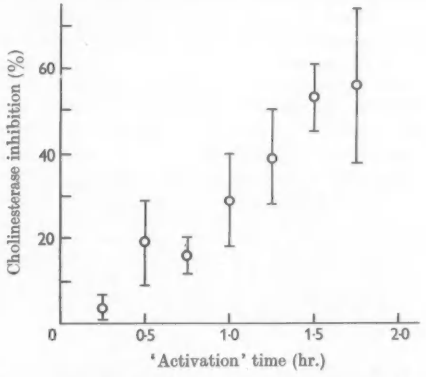

Fig. 5. Effect of period of contact between cockroach gut and schradan *in vitro* on schradan 'activation', as assayed by inhibition of erythrocyte cholinesterase by the activated schradan. Each point is an average of four experiments, each with a single gut. Vertical lines show standard deviation.

Another problem was that SKF 525A has anticholinesterase activity. The I_{50} (concentration for 50% inhibition) was 6·3 μM for human plasma, 0·32 mM for housefly head and 2·5 mM for laked human erythrocytes. The last-named were therefore used subsequently with SKF 525A. With plasma it was shown that inhibition was independent of time of incubation with the plasma, between 20 and 70 min. The inhibition is thus not progressive as with organophosphates, but time-independent.

Table 4 shows that schradan 'activation' by cockroach gut was substantially inhibited by SKF 525A even at 10^{-5} M. Unfortunately the extent of inhibition cannot be quantitatively stated, since no figure is available for the anticholinesterase potency of the 'activation' product, hydroxymethylschradan, against erythrocyte cholinesterase. If it is substantially the same as for plasma cholinesterase (O'Brien & Spencer, 1955), one may calculate that 10^{-5} M-SKF 525A inhibited production of hydroxymethylschradan by about 65%.

DISCUSSION

These findings suggest (but do not prove) that SKF 525A fails to protect against parathion poisoning because it has two contrary actions: it inhibits the production of paraoxon from parathion (as judged by studies *in vitro*) but also inhibits the degradation of paraoxon. The same phenomena are observed in the cockroach and the mouse.

From the limited data available, SKF 525A appears to be an inhibitor of 'activation' of phosphorothionates in general. Yet it sometimes antagonizes, sometimes synergizes and sometimes has no effect upon phosphorothionate poisoning, according to the species and the phosphorothionate that are examined. Presumably this variation reflects variations in the relative potency of its inhibition of activation and degradation in each case.

Table 4. *Effect of SKF 525A on schradan activation by cockroach guts*

The figures were obtained by reference to a control treated with the same level of SKF 525A. The indicated concentration of SKF 525A during incubation with the gut was 2·7 times that in the subsequent incubation with cholinesterase. For details see Methods.

Concn. of SKF 525A (M)	Percentage erythrocyte-cholinesterase inhibition caused by 'activated' schradan
0	59
10^{-3}	14
10^{-4}	23
10^{-5}	29

The only non-metallic inhibitor of a paraoxon-degrading enzyme reported so far is ethylenediaminetetra-acetic acid (Main, 1960). SKF 525A should prove a valuable tool in assessing the importance of paraoxonase and related enzymes, and in distinguishing between the various enzymes that degrade organophosphates. Its use in studies of phosphorothionate activation must clearly be limited to those cases where appropriate degrading enzymes are absent; such conditions have not yet been achieved.

The activating systems of the cockroach and mouse are virtually identical in their sensitivity to SKF 525A (Table 3). The demonstration that the 'activation' of schradan by cockroach gut is inhibited by SKF 525A, as had been shown for 'activation' by mouse liver (Davison, 1955), is a further point of similarity. Nevertheless, there is evidence that the systems are in fact substantially different, particularly in their cofactor requirements when broken-cell preparations are used (O'Brien, 1956).

One feature of this study is the lack of correlation between toxicity and the total body level of paraoxon. For instance, Figs. 1 and 2 show that the agent SKF 525A has a marked effect upon the paraoxon level of mice poisoned either with parathion or paraoxon. Yet the agent has a negligible effect on the toxicity of these organophosphates. This finding throws some doubt on the validity of conclusions about toxicity drawn from observations on whole-body levels of toxic compound, as was extensively done by Krueger *et al.* (1960), and as is implicit in the present study. Instead one should perhaps study the concentration of toxic compound in, say, the nervous system. Compounds of this type are particularly likely to diffuse rapidly into lipoidal tissue, such as the nervous system, by virtue of their high lipid–water partition coefficients. We have observed partition coefficients between triacetin and water of 6900 for parathion, 37 for paraoxon and 39 for malathion.

SUMMARY

1. SKF 525A (2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride) protects mice against poisoning by the three phosphorodiamidic compounds tested. Of the eight phosphorothionates tested, protection against dimethoate only was observed.

2. With houseflies and American cockroaches, SKF 525A gave no protection against any phosphorothionates.

3. SKF 525A inhibited the conversion of parathion into paraoxon by mouse-liver slices or cockroach guts.

4. SKF 525A inhibited the 'activation' of schradan by cockroach guts, as had previously been shown for rat-liver preparations.

5. With mice and cockroaches *in vivo*, SKF 525A increased the concentration of paraoxon caused by the injection of parathion, and reduced the degradation of injected paraoxon.

6. It was concluded that the diverse effects of SKF 525A on organophosphate toxicity in various species were due to the variations in the importance of inhibition of 'activating' as opposed to degrading enzymes.

Most of the toxicity studies were carried out in Dr J. E. Casida's laboratory in the Department of Entomology, University of Wisconsin, by Miss B. Gordon, and were supported in part by a grant from the U.S. Public Health Service, National Institutes of Health. Thanks are also due to Mr L. G. Crawford for his able technical assistance in the work at London.

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Mitochondria of the Ehrlich Ascites-Tumour Cell

2. DIPHOSPHOPYRIDINE NUCLEOTIDE- AND TRIPHOSPHOPYRIDINE NUCLEOTIDE-DEPENDENT OXIDATION OF *ISO*CITRATE*

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Studies of oxidative phosphorylation in Ehrlich ascites-cell mitochondria with α-oxoglutarate, L-glutamate, succinate and ascorbate as substrates were reported by Hawtrey & Silk (1960*a*). The present paper describes studies of *iso*citrate oxidation in the same tumour mitochondria and amplifies the findings of a preliminary report (Hawtrey & Silk, 1960*b*).

The mechanism of *iso*citrate oxidation has been investigated in considerable detail in normal-cell mitochondria, but little is known about the pathways which operate in malignant cells.

In rat-liver mitochondria and rat-heart sarcosomes the cofactor of *iso*citric dehydrogenase has been shown to be triphosphopyridine nucleotide (Kaplan, Swartz, Frech & Ciotti, 1956; Purvis, 1958*a*, *b*), although Ernster (1959) maintains that a diphosphopyridine nucleotide-dependent *iso*citric dehydrogenase is also present. Vignais, Vignais & Bartley (1957) have reported P:O ratios for diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent *iso*citric dehydrogenases in the presence of intact rat-liver mitochondria, and both diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent enzymes have been demonstrated in ox-adrenal mitochondria (Grant & Mongkolkul, 1958).

The cofactor requirement of *iso*citric dehydrogenase in tumour-cell mitochondria does not appear to have been correspondingly well investi-

* Part 1: Hawtrey & Silk (1960*a*).

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gated, although Reinafarje & Potter (1957) and Williams-Ashman (1953) have demonstrated a requirement for triphosphopyridine nucleotide in the Novikoff and Ehrlich tumours respectively.

Studies concerning pyridine nucleotide metabolism associated with *iso*citrate oxidation have indicated the presence of a transhydrogenase interconverting reduced triphosphopyridine nucleotide and diphosphopyridine nucleotide in normal-cell mitochondria (Kaplan *et al.* 1956; Purvis, 1958*a*, *b*), but this enzyme appears to be absent from Novikoff hepatoma-cell mitochondria (Reinafarje & Potter, 1957). Williams-Ashman (1953), on indirect evidence, using acetone extracts of whole tumour cells, concluded that the transhydrogenase enzyme is also absent from the Ehrlich ascites tumour.

The present investigation was undertaken to establish the pathway of *iso*citrate oxidation directly in Ehrlich ascites-cell mitochondria and to determine the cofactor requirement of *iso*citric dehydrogenase in these particulate preparations. Pyridine nucleotide metabolism and phosphorylations associated with *iso*citrate oxidation have also been investigated.

MATERIALS AND METHODS

Ehrlich ascites tumour. This tumour was kindly supplied by Dr K. Sugiura of the Sloan-Kettering Institute, New York, and has been maintained in Swiss albino mice which were used between the seventh and tenth day after inoculation.

Hexokinase. Crystalline material was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. The enzyme was assayed by measurement of hexose disappearance according to Somogyi (1952), and by measurement of the disappearance of acid-labile phosphorus according to Crane & Sols (1955). Preparations had an activity of approx. 150 units/mg.

Alcohol dehydrogenase. This was supplied by General Biochemicals Inc. and was assayed according to the method of Racker (1955). Preparations had a specific activity of $1{\cdot}1 \times 10^5$ units.

Cytochrome c. A 1% (w/v) solution in aq. 0·09% NaCl was supplied by General Biochemicals Inc., and contained 0·587 μmole/ml. when assayed according to Umbreit, Burris & Stauffer (1957).

Adenosine 5′-phosphate and adenosine diphosphate. These were used as supplied by General Biochemicals Inc., and by the H.M. Chemical Co. of Los Angeles, Calif., U.S.A., respectively.

Amytal. Amytal (sodium salt of 5-*iso*amyl-5-ethylbarbituric acid) was supplied by Eli Lilly and Co., Indianapolis, Ind., U.S.A. Solutions were prepared in water.

Antimycin A. This was obtained from the Wisconsin Alumni Research Foundation, Madison, Wis., U.S.A. Solutions were prepared in aq. 50% ethanol and were used immediately.

Diphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed 85% pure by a modification of the method of Brodie (1955) with 10 mg. of $Na_2S_2O_4$ per 1·0 ml. of 0·2 M-Na_2HPO_4 as the reducing solution.

Triphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed as 70% pure according to the method of De Moss (1955).

D-*Glucose* 6-*phosphate.* This was supplied as the barium salt by General Biochemicals Inc., and converted into the sodium salt by treatment with Na_2SO_4 according to the method of De Moss (1955).

D-*Glucose* 6-*phosphate dehydrogenase.* The enzyme was used as supplied by General Biochemicals Inc.

*iso*Citrate. This was prepared from DL-*iso*citric acid lactone supplied by General Biochemicals Inc. The lactone (17·4 mg.) in 0·6 ml. of 0·5 N-KOH was heated on a boiling-water bath for 10 min., cooled, neutralized to pH 7·4 with dil. HCl and adjusted to a final volume of 1·0 ml. with water. Solutions were stored at −30° and used within 7 days.

Dioxan. The AnalaR grade of British Drug Houses Ltd. was purified by refluxing with excess of Na before distillation into dark-brown bottles for storage at −30°.

Ovalbumin. This was a twice recrystallized and freeze-dried sample obtained from L. Light and Co. Ltd., Colnbrook, Bucks, England.

Other chemicals. Nicotinamide, A.R. grade, was supplied by E. Merck, Germany. Ethanol, A.R. grade, and digitonin, A.R. grade, were supplied by British Drug Houses Ltd. Ordinary grades of 2:6-dichlorophenol-indophenol and 2:4-dinitrophenol were obtained from British Drug Houses Ltd. Chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl) phenothiazine hydrochloride] was supplied by May and Baker Ltd. All other chemicals were of analytical-reagent grade.

Isolation of Ehrlich ascites-cell mitochondria. These were isolated according to the procedure of Hawtrey & Silk (1960*a*). The pH of the final suspension in 0·25 M-sucrose was 6·9 as measured with the glass electrode.

Depletion of pyridine nucleotides. Mitochondria were depleted of their pyridine nucleotide content in one of two ways: (1) Incubation with sodium potassium phosphate buffer at 30°. To 1·6 ml. of mitochondrial suspension in 0·25 M-sucrose was added 1·6 ml. of 0·25 M-sucrose in 0·06 M-sodium potassium phosphate buffer, pH 7·4. The mixture was gently homogenized with five passes of the tight plunger in a small Dounce homogenizer (supplied by Blaessig Glass Specialties, Rochester, N.Y., U.S.A.). After homogenization, the mitochondrial suspension was incubated aerobically on a Warburg bath for 35–40 min. at 30°, and then placed immediately in ice. (2) Depletion by aging. The mitochondrial suspension in 0·25 M-sucrose was aged for 5–6 hr. at 0°.

Preparation of rat-liver mitochondria depleted of pyridine nucleotides. Rat-liver mitochondria were isolated in 0·44 M-sucrose according to the method of Dounce, Witter, Monty, Pate & Cottone (1955). The particles were twice washed in 0·25 M-sucrose and were depleted of pyridine nucleotides by incubation in 0·05 M-sucrose with 0·03 M-phosphate buffer (pH 7·4) for 60 min. at 30°. A final suspension was prepared in 0·25 M-sucrose and stored at 0°.

Mitochondrial-protein content. This was determined according to the biuret method of Cleland & Slater (1953) with an ovalbumin standard.

Digitonin treatment of mitochondria. Mitochondria were first depleted of pyridine nucleotides by incubation with

phosphate buffer. To 0·6 ml. of the final suspension in 0·25 M-sucrose and 0·03 M-phosphate buffer (pH 7·4) was added 0·6 ml. of a freshly prepared 1% digitonin solution. The preparation was thoroughly mixed and kept for 20 min. at 0° with occasional stirring. It was then assayed immediately. The mitochondria were apparently solubilized to some extent by digitonin treatment as evidenced by a clearing of the suspension.

Spectrophotometric assays with 2:6-dichlorophenol-indophenol and cytochrome c. Measurements were made in the 1 cm. cells of a Beckman Model DU spectrophotometer. The medium contained the following basic additions in a final volume of 3·0 ml.: 0·10 M-sodium potassium phosphate buffer, pH 7·4; mM-KCN; 3 mM-$MgCl_2$; 2·3 mM-DL-*iso*citrate (potassium salt; pH 7·4); 0·02 M-nicotinamide; 36 μM-2:6-dichlorophenol-indophenol or 40 μM-cytochrome *c*; 1000–1500 μg. of diphosphopyridine nucleotide (DPN); 400–1000 μg. of triphosphopyridine nucleotide (TPN); Ehrlich ascites-cell mitochondria (0·20–0·31 mg. of protein) or rat-liver mitochondria (0·92 mg. of protein), both depleted of pyridine nucleotides. The following additions were made as required: reduced diphosphopyridine nucleotide (DPNH), generated from 1000 μg. of DPN by addition of 0·04 ml. of ethanol and 0·5 mg. of alcohol dehydrogenase to the medium; reduced triphosphopyridine nucleotide (TPNH), generated from 400–1000 μg. of TPN by addition of 1·7 mM-glucose 6-phosphate and 1·0 mg. of glucose 6-phosphate dehydrogenase to the medium; 0·19 M-dioxan; 0·10 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetate buffer (various pH values) in place of phosphate buffer; 2 mM-amytal; 0·2 mM-chlorpromazine; 0·2 mM-2:4-dinitrophenol; 5 μg. of antimycin A.

Cuvettes were examined visually after each reaction to determine whether agglutination or precipitation of the mitochondria had occurred. Reduction of 2:6-dichlorophenol-indophenol was followed at 600 mμ, and reduction of cytochrome *c* at 550 mμ.

Measurement of oxygen uptake and oxidative phosphorylation. The following medium in a volume of 2·36 ml. was placed in the main compartment of each Warburg flask (molarities refer to the final volume of 3·0 ml. after addition of the side-arm contents and mitochondrial suspension): 0·045 M-sodium potassium phosphate buffer, pH 7·4; 34 mM-D-glucose; 15 mM-KF; 8 mM-$MgCl_2$; mM-adenosine 5′-phosphate (AMP); 2·5 mM-adenosine diphosphate (ADP); 2 mM-ethylenediaminetetra-acetic acid (EDTA); 0·01 M-KCl; 62 mM-sucrose; 9·8 μM-cytochrome *c*; 3·3 mM-DL-*iso*citrate; 1·2 mM-DPN or TPN as required; 0·19 M-dioxan as required. (All substrates and cofactors were neutralized to pH 7·4 with KOH and stored at −30° before use.) The side arms of each flask contained 70% of the total D-glucose, together with 1–3 mg. of crystalline hexokinase in a volume of 0·24 ml.

To each Warburg vessel was added 0·4 ml. of the final suspension of mitochondria depleted of pyridine nucleotides, giving a protein concentration of between 0·60 and 0·88 mg./ml. after addition of the side-arm contents. The centre well of each flask contained 0·2 ml. of 40% (w/v) KOH for CO_2 absorption, and the gas phase was air. Uptake of O_2 was measured for 25–30 min. after equilibration for 9–12 min. at 30°. The reaction was stopped by addition of trichloroacetic acid and phosphate uptake was determined by the method of Fiske & Subbarow (1925) on cold trichloroacetic acid filtrates.

RESULTS

Cofactor requirement of isocitric dehydrogenase. This was investigated with mitochondria depleted of their endogenous pyridine nucleotides by incubation in phosphate buffer. The effects of added DPN, TPN and a mixture of DPN + TPN on *iso*citrate oxidation were measured with 2:6-dichlorophenol-indophenol as electron acceptor (cf. Hawtrey & Silk, 1960*b*; Fig. 1). *iso*Citrate was not oxidized in the absence of added DPN or TPN, as expected, with mitochondria depleted of pyridine nucleotides, but oxidation occurred rapidly in the presence of DPN and at a slower rate in the presence of TPN. The initial rate of *iso*citrate oxidation with a mixture of DPN + TPN together was the same as that with DPN alone, but a slight decrease was observed after about 6 min.

DPNH produced *in situ* by the alcohol dehydrogenase reaction:

$$C_2H_5{\cdot}OH + DPN^+ \rightleftharpoons CH_3{\cdot}CHO + DPNH + H^+$$

was very rapidly oxidized with 2:6-dichlorophenol-indophenol as electron acceptor.

TPNH, produced *in situ* by the glucose 6-phosphate dehydrogenase reaction:

$$\text{Glucose 6-phosphate} + TPN^+ \Downarrow \text{6-phosphogluconate} + TPNH + H^+$$

was oxidized with 2:6-dichlorophenol-indophenol as acceptor, but at a much slower rate than DPNH.

Externally produced TPNH was oxidized at approximately twice the rate of TPNH generated from *iso*citrate oxidation in the presence of TPN.

An important finding with regard to the question of transhydrogenase activity in Ehrlich mitochondria was that added DPN did not increase the oxidation rate of externally generated TPNH.

Results obtained with cytochrome *c* as electron acceptor are shown in Fig. 1. The curves occupy the same relative positions as those obtained with 2:6-dichlorophenol-indophenol, except that *iso*citrate was more rapidly oxidized in the presence of DPN + TPN than in the presence of DPN alone. Cytochrome *c* reduction was initially rapid with *iso*citrate in the presence of DPN but the rate decreased considerably after 2 min. This was consistently observed with different batches of mitochondria. In correspondence with the results with 2:6-dichlorophenol-indophenol as electron acceptor, externally generated TPNH was oxidized at a greater rate than the TPNH resulting from *iso*citrate oxidation in the presence of TPN.

The effect of cofactors on *iso*citrate oxidation was also examined with molecular oxygen as acceptor (cf. Hawtrey & Silk, 1960*b*; Fig. 2). Oxygen uptake was considerably greater with DPN

as cofactor than with TPN. In the presence of DPN + TPN, *iso*citrate oxidation occurred at a slower rate than with DPN alone. This finding parallels the result with 2:6-dichlorophenol-indophenol as electron acceptor.

In contrast with the results with 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors, it was observed that, with molecular oxygen as terminal acceptor, externally generated TPNH was oxidized at a much slower rate than the TPNH resulting from *iso*citrate oxidation in the presence of TPN. Mitochondria containing 1·91 mg. of protein absorbed only 0·51 μg.atom of oxygen in the presence of TPNH externally generated from 1·2 mM-TPN, whereas 3·41 μg.atoms of oxygen were absorbed in the presence of TPNH generated from *iso*citrate and an equivalent concentration of TPN.

Variation in diaphorase activity with coenzyme concentration. Mitochondria depleted of pyridine nucleotides by incubation in phosphate buffer were assayed for diaphorase activity by using various concentrations of DPN and TPN, with 2:6-dichlorophenol-indophenol as electron acceptor. Fig. 2 shows that up to a concentration of 0·67 mM-DPN the rate of increase in dye reduction was proportional to the amount of cofactor present. At higher concentrations of DPN a diminishing rate of increase was observed. Fig. 3 shows that with TPN as coenzyme the rate of dye reduction became maximal at a concentration of 0·3 mM-TPN.

The effect of TPN concentration on TPN–*iso*citrate–cytochrome *c* reductase activity is shown in Fig. 4. Maximum activity occurred at a concentration of 75 μM-coenzyme, with inhibition of dye reduction above this molarity.

An increase in DPN concentration from 0·24 mM to 2·4 mM had no effect on the oxygen uptake of mitochondria which had been depleted of pyridine nucleotides by aging for 5 hr. in sucrose at 0° (Table 1).

Since albumins are reported to have the property of adsorbing inhibitors of respiration and oxidative phosphorylation (Wojtczak & Wojtczak, 1959), the oxygen uptake of pyridine nucleotide-depleted Ehrlich mitochondria was measured with 1·2 mM-DPN as cofactor in the presence of ovalbumin (10 mg./ml. of medium). A 13% decrease in DPN-linked activity was observed (Table 1).

Effect of pH *on diaphorase activity.* The effect of variation in pH on the DPN- and TPN-linked

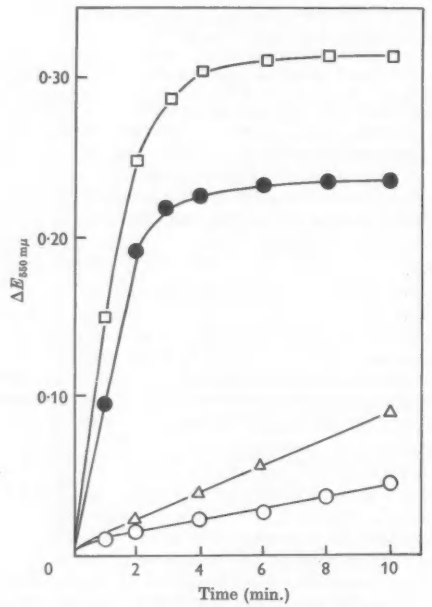

Fig. 1. Reduction of cytochrome *c* by pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria in the presence of *iso*citrate and added cofactors. Cuvettes contained 0·26 mg. of mitochondrial protein. ○, *iso*Citrate + TPN; △, TPNH; ●, *iso*citrate + DPN; □, *iso*citrate + DPN + TPN.

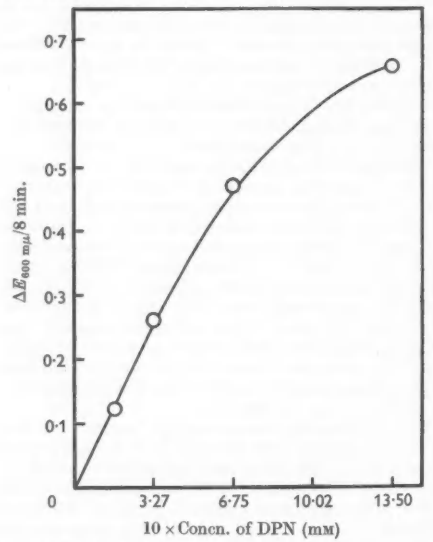

Fig. 2. Effect of added DPN on the rate of *iso*citrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0·31 mg. of mitochondrial protein.

diaphorase activities associated with *iso*citrate oxidation was measured in tris–acetate buffers with 2:6-dichlorophenol-indophenol as electron acceptor. Mitochondria were depleted of pyridine nucleotides by incubation with phosphate buffer. Results in Fig. 5 show that the DPN-linked *iso*citric diaphorase has a pH optimum of 7·1 with a fall in activity on either side of this value. The TPN-linked *iso*citric-diaphorase system showed a pH optimum at 6·2 with an inflexion at pH 7·8. The activity at pH 6·2 was approximately two-thirds that of the DPN-linked diaphorase at pH 7·1. Table 2 shows that in phosphate buffer at pH 7·4 both DPN- and TPN-dependent diaphorases were almost twice as active as in tris–acetate buffer at the same pH.

Effect of aging on diaphorase activity. Mitochondria which had been depleted of pyridine nucleotides by incubation with phosphate buffer at 30°, were aged over 84 hr. at 0° in 0·25 M-sucrose plus 0·03 M-phosphate buffer, pH 7·4. Samples of the mitochondrial suspension were removed, after intervals of 1, 24, 50, 72 and 84 hr., for assay of diaphorase activity with 2:6-dichlorophenol-indophenol as electron acceptor. The results in Table 3 show that DPN-dependent diaphorase activity

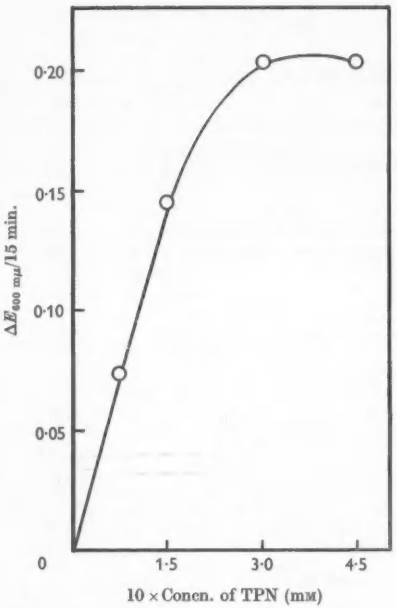

Fig. 3. Effect of added TPN on the rate of *iso*citrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0·31 mg. of mitochondrial protein.

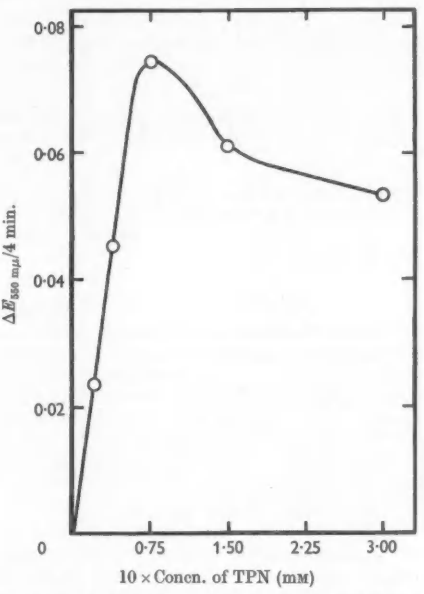

Fig. 4. Effect of added TPN on the rate of *iso*citrate oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with cytochrome *c* as electron acceptor. Cuvettes contained 0·31 mg. of mitochondrial protein.

Table 1. *Effect of added diphosphopyridine nucleotide on* isocitrate *oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Oxygen uptake was measured in Warburg manometers at 30°. Experimental conditions were as in the text. Ovalbumin, 10 mg./ml. of incubation medium.

System oxidized	Method of nucleotide depletion	Protein (mg./flask)	O_2 uptake (μg.atoms)
DPN (2·4 mM) + *iso*citrate	Aging for 5 hr. in 0·25 M-sucrose at 0°	2·63	7·92
DPN (0·24 mM) + *iso*citrate		2·63	8·10
DPN (1·2 mM) + *iso*citrate	Incubation with phosphate buffer at 30°	2·02	9·64
DPN (1·2 mM) + *iso*citrate + ovalbumin		2·02	8·44

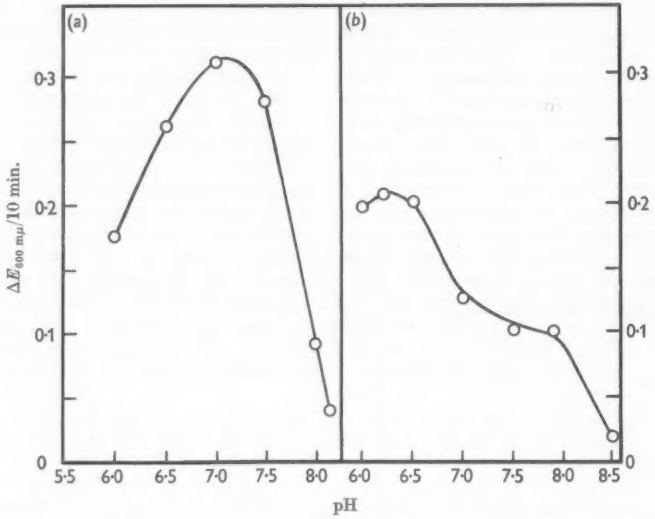

Fig. 5. Effect of pH on (*a*) DPN-dependent and (*b*) TPN-dependent *iso*citric-dehydrogenase activity in Ehrlich ascites-cell mitochondria. The buffers were tris–acetate with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0·24 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 1500 μg. of DPN or 400 μg. of TPN.

Table 2. *Diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent* isocitric-diaphorase *activities of pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria in phosphate and tris–acetate buffers*

Experimental conditions were as in the text. Cuvettes contained 0·18 mg. of mitochondrial protein and 1500 μg. of DPN or 400 μg. of TPN. The electron acceptor was 2:6-dichlorophenol-indophenol.

Buffer system	$\Delta E_{600\ m\mu}$/10 min. DPN + *iso*citrate	TPN + *iso*citrate
0·1 M-KH_2PO_4–Na_2HPO_4 (pH 7·4)	0·478	0·172
0·1 M-Tris–acetate (pH 7·4)	0·283	0·100

dropped approximately 70 % on storage for 84 hr. at 0° whereas that of the TPN-dependent enzyme system remained practically unchanged.

Phosphorylation associated with isocitrate *oxidation.* Results in Table 4 show that *iso*citrate oxidation is coupled to phosphorylation in the presence of both DPN and TPN as cofactor. In mitochondria completely depleted of pyridine nucleotides by incubation with phosphate buffer, P:O ratios associated with the DPN pathway lay between 1·11 and 1·31, whereas P:O ratios of 1·07 and 1·31 were measured with TPN as cofactor. P:O ratios of 0·6–0·7 were measured for the DPN pathway in mitochondria depleted of pyridine nucleotides by aging for 5½ hr. in 0·25 M-sucrose at 0°. In no experiment was a P:O ratio greater than 1·31 observed for *iso*citrate oxidation.

Table 3. *Effect of aging on the diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent* isocitric-diaphorase *activities of pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Mitochondria were aged by storage in 0·25 M-sucrose at 0° for the times shown. Experimental conditions were as in the text. Cuvettes contained 0·26 mg. of mitochondrial protein and 1500 μg. of DPN or 400 μg. of TPN. The electron acceptor was 2:6-dichlorophenol-indophenol.

Time stored at 0° (hr.)	$\Delta E_{600\ m\mu}$/12 min. DPN + *iso*citrate	TPN + *iso*citrate
1	0·548	0·200
24	0·404	0·190
50	0·306	0·188
72	0·228	0·196
84	0·167	0·191

Effect of respiratory-chain inhibitors and uncouplers on electron transport. The effects of amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on both DPN- and TPN-linked *iso*citrate oxidations were assayed with cytochrome *c* as electron

Table 4. *Phosphorylations associated with diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent* isocitrate *oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Experimental conditions were as in the text. Results are shown for mitochondria depleted of endogenous pyridine nucleotides by two different methods. Temp., 30°.

Method of nucleotide depletion	Cofactor DPN (m-moles)	Cofactor TPN (m-moles)	ΔO (μg.atoms)	ΔP (μmoles)	P:O
Incubated with phosphate buffer for 35–40 min. at 30°, then stored at 0°	—	1·2	3·00	3·22	1·07*
	—	1·4	2·46	3·22	1·31
	1·2	—	8·24	9·68	1·18*
	1·4	—	8·17	9·07	1·11
	2·4	—	5·28	6·93	1·31
Aged for 5½ hr. in 0·25 M-sucrose at 0°	2·4	—	8·22	4·65	0·57
	2·8	—	8·55	5·60	0·66

* Similar results with three other mitochondrial preparations. Two further preparations showed satisfactory oxygen uptake but were devoid of phosphorylation activity.

Table 5. *Effect of respiratory chain inhibitors and uncouplers on electron transport associated with diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent* isocitrate *oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria*

Experimental conditions were as in the text. Cuvettes contained 0·35 mg. of mitochondrial protein and 1·0 mg. of DPN or TPN. Values of $\Delta E_{550\,m\mu}$ are shown for the initial 2 min. Curves remained practically linear over 15 min. except with antimycin A. The election acceptor was cytochrome *c*.

Inhibitor or uncoupler	Concn. (mM)	$\Delta E_{550\,m\mu}$ over initial 2 min. DPN + *iso*citrate	$\Delta E_{550\,m\mu}$ over initial 2 min. TPN + *iso*citrate
Control	—	0·239	0·030
Amytal	2·0	0·162	0·020
Chlorpromazine	0·2	0·237	0·057
2:4-Dinitrophenol	0·2	0·227	0·014
Antimycin A (5 μg.)	—	—	0·003 (→ 0·030 after 15 min.)

acceptor. Mitochondria were completely depleted of pyridine nucleotides by incubation in phosphate buffer, but were not preincubated with the compounds under test. The reaction was assayed immediately after mixing with the inhibitor or uncoupler concerned. Curves were linear during the first 2 min. and remained practically so over 15 min. except in the case of antimycin A. Table 5 shows that 2 mM-amytal caused a 30 % decrease in both DPN- and TPN-dependent *iso*citrate oxidation. Chlorpromazine at a concentration of 0·2 mM had no effect on DPN-linked *iso*citrate oxidation, but caused an approximately twofold increase in TPN-dependent diaphorase activity. The uncoupling agent 2:4-dinitrophenol was also without effect on the DPN diaphorase, but decreased the activity of TPN-linked *iso*citrate oxidation by approximately 50 %. Antimycin A was tested only on the TPN-dependent system, which it failed to inhibit except during an initial period of 2 min. Thereafter cytochrome *c* reduction increased until the control value was reached after 15 min.

With 2:6-dichlorophenol-indophenol as electron acceptor, 2 mM-amytal had no effect on the rate of DPN-dependent *iso*citrate oxidation.

Effect of dioxan on electron transport. Oestrone and oestradiol have been shown to act as cofactors for the transhydrogenase enzymes in the high-speed supernatant fraction of rat-liver homogenates (Talalay & Hurlock, 1958). Solutions of these steroids in dioxan stimulated diaphorase activity associated with both DPN- and TPN-linked *iso*citrate oxidation in Ehrlich mitochondria, but to no greater extent than an equivalent concentration of pure dioxan. The effect of this solvent on *iso*citrate oxidation was accordingly examined in greater detail with mitochondria depleted of pyridine nucleotides by incubation in phosphate buffer.

Fig. 6 shows the stimulating effect of pure dioxan on both DPN- and TPN-diaphorase activities with 2:6-dichlorophenol-indophenol as electron acceptor. The TPN-dependent activity was enhanced to a greater extent than the DPN-dependent. Separate experiments showed that maximum stimulation of activity occurred at a concentration of 0·19 M-dioxan.

Table 6 shows that, in contrast with the results with 2:6-dichlorophenol-indophenol as electron acceptor, dioxan caused a considerable lowering in

the activities of both DPN- and TPN-linked *iso*citrate oxidations when either cytochrome c or molecular oxygen was used as the terminal electron acceptor. This difference in effect is illustrated in Fig. 7 with reference to TPN-linked *iso*citrate oxidation. With 0·19M-dioxan, which affords maximum TPN-diaphorase activity when 2:6-dichlorophenol-indophenol is the electron acceptor, the activity with oxygen as acceptor was at its lowest level.

The effect of dioxan on *iso*citrate oxidation in normal rat-liver mitochondria was investigated for comparison with its effect on Ehrlich tumour mitochondria. The rat-liver mitochondria were depleted of pyridine nucleotides by incubation in phosphate buffer.

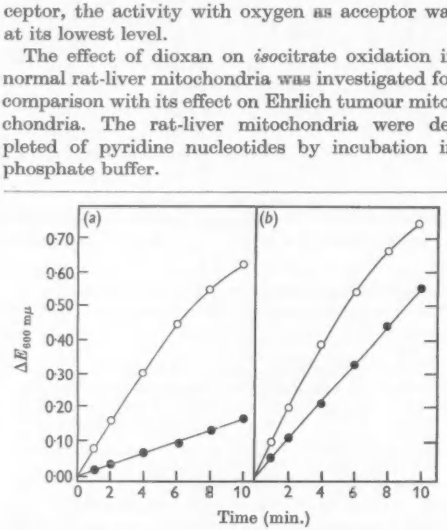

Fig. 6. Effect of 0·19M-dioxan on (*a*) TPN-dependent and (*b*) DPN-dependent *iso*citrate oxidation in Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0·20 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 1000 μg. of DPN or 400 μg. of TPN. ○, Dioxan present; ●, dioxan absent.

Table 6. *Effect of dioxan on diphosphopyridine nucleotide-dependent and triphosphopyridine nucleotide-dependent* isocitrate *oxidation in pyridine nucleotide-depleted Ehrlich ascites-cell mitochondria with cytochrome* c *and molecular oxygen as terminal electron acceptors*

Experimental conditions were as in the text. Cuvettes contained: 40 μM-cytochrome *c*; 0·20 mg. of mitochondrial protein; 1000 μg. of DPN or TPN; and 0·19M-dioxan. Oxygen uptake was measured in Warburg manometers at 30°. Flasks contained: 1·90 mg. of mitochondrial protein; 1·2 mM-DPN or -TPN; and 0·19M-dioxan.

Terminal electron acceptor...	Cytochrome c	Oxygen
System oxidized	$\Delta E_{550m\mu}$/2 min.	(μg.atoms/mg. of protein/hr.)
DPN + *iso*citrate	0·165	10·13
DPN + *iso*citrate + dioxan	0·052	4·13
TPN + *iso*citrate	0·024	3·21
TPN + *iso*citrate + dioxan	0·010	1·70

Table 7 shows that with 2:6-dichlorophenol-indophenol as electron acceptor, 0·19M-dioxan caused a depression of 40% in diaphorase activity associated with TPN-dependent *iso*citrate oxidation. The rate of dye-reduction in the presence of DPN was the same as the control value for *iso*citrate without cofactors, and was unaffected by

Table 7. *Effect of dioxan on* isocitrate *oxidation in pyridine nucleotide-depleted rat-liver mitochondria with* 2:6-*dichlorophenol-indophenol as electron acceptor*

Preparation of mitochondria and experimental conditions were as in the text. Cuvettes contained: 0·92 mg. of mitochondrial protein; 400 μg. of DPN or TPN; and 0·19M-dioxan. TPNH was externally generated from 400 μg. of TPN by the glucose 6-phosphate-dehydrogenase reaction.

System oxidized	$\Delta E_{600\ m\mu}$/8 min.
Control. *iso*Citrate (no cofactor)	0·010
DPN + *iso*citrate	0·016
DPN + *iso*citrate + dioxan	0·018
TPN + *iso*citrate	0·380
TPN + *iso*citrate + dioxan	0·223
DPN + TPN + *iso*citrate	0·464
DPN + TPN + *iso*citrate + dioxan	0·514
TPNH	0·386
TPNH + dioxan	0·483

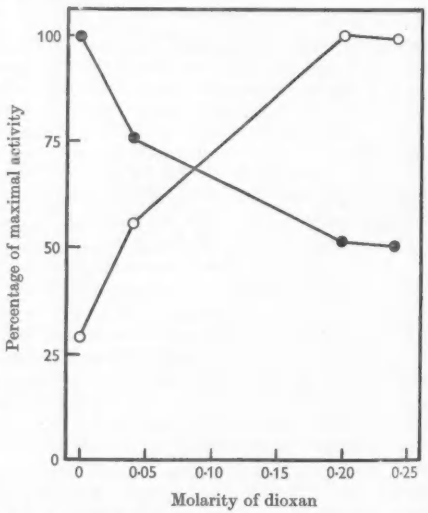

Fig. 7. Effect of dioxan concentration on TPN-dependent *iso*citrate oxidation in Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol (○) and molecular oxygen (●) as electron acceptors. Cuvettes contained 0·20 mg. of mitochondrial protein (depleted of pyridine nucleotides) and 400 μg. of TPN. Oxygen uptake was measured in Warburg manometers containing 1·2 mM-TPN.

addition of dioxan. Addition of DPN to the TPN-dependent *iso*citrate-oxidase system caused a 21% increase in diaphorase activity, which was enhanced a further 14% by addition of 0·19M-dioxan. Externally added TPNH was oxidized at the same rate as TPNH generated from *iso*citrate oxidation in the presence of TPN, but dioxan, in contrast with its depressant effect on TPN-dependent *iso*citrate oxidation, caused a stimulation of 25% in the oxidation rate of externally added TPNH.

Effect of digitonin on electron transport. Both DPN- and TPN-linked *iso*citrate-diaphorase activities were assayed in digitonin-treated mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. The results are shown in Fig. 8, together with control curves obtained with the same batch of mitochondria which had been depleted of pyridine nucleotides but which had not been treated with digitonin.

Digitonin treatment stimulated DPN-dependent *iso*citrate-diaphorase activity, but was without effect on the TPN-linked system.

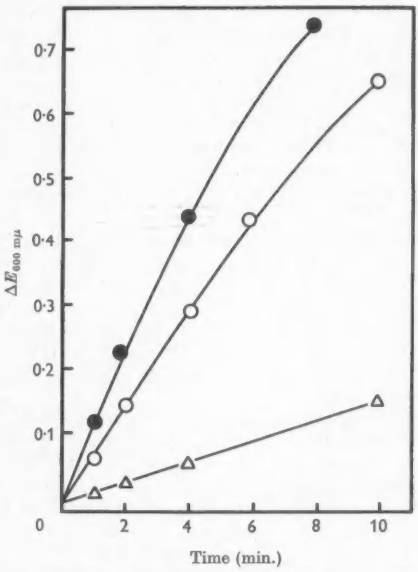

Fig. 8. Effect of digitonin on DPN-dependent and TPN-dependent *iso*citrate oxidation in Ehrlich ascites-cell mitochondria with 2:6-dichlorophenol-indophenol as electron acceptor. Cuvettes contained 0·29 mg. of mitochondrial protein (depleted of pyridine nucleotide content and pretreated with digitonin) and 1500 μg. of DPN or 400 μg. of TPN. ●, DPN and digitonin; ○, DPN; △, TPN with and without digitonin.

DISCUSSION

It may be concluded from this study that mitochondria of the Ehrlich ascites cell possess both DPN- and TPN-dependent *iso*citric-dehydrogenase enzyme systems. This is in contrast with rat-liver mitochondria, which have been shown to contain only a TPN-specific *iso*citric dehydrogenase (Table 7) in agreement with Kaplan *et al.* (1956) and with Purvis (1958*a*, *b*). Of the two *iso*citrate oxidation pathways in Ehrlich mitochondria, the DPN-dependent system is some three to four times as active as its TPN counterpart. This may possibly be connected with the observation that TPN concentrations are very low in tumour cells [Wenner & Weinhouse (1953) and Glock & McLean (1957)].

The observation that *iso*citrate oxidation with all electron acceptors is more rapid in the presence of DPN + TPN than in the presence of TPN alone, probably represents an additive effect since separate DPN- and TPN-linked pathways exist in Ehrlich mitochondria. If separate pathways did not exist, then it would be necessary to postulate the operation of a transhydrogenase enzyme transferring hydrogen from TPNH to DPN, yielding DPNH, which is very rapidly oxidized (cf. Hawtrey & Silk, 1960*b*; Fig. 1). Since addition of DPN did not stimulate the oxidation rate of externally added TPNH (Hawtrey & Silk, 1960*b*), it may be concluded that the transhydrogenase pathway is either absent or of little importance in Ehrlich mitochondria. The necessity for a pyridine nucleotide transhydrogenase is apparently obviated by the existence of a direct pathway to oxygen for reoxidation of TPNH generated from TPN during *iso*citrate oxidation. On different experimental grounds Williams-Ashman (1953) also concluded that transhydrogenase activity is absent in Ehrlich cells, whereas Reinafarje & Potter (1957) failed to detect the enzyme in Novikoff hepatoma cells. Findings regarding transhydrogenase activity in a variety of cell types have been summarized by Kit & Griffin (1958).

Since external TPNH is more rapidly oxidized with 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors than TPNH generated from TPN during *iso*citrate oxidation, it would seem that the dehydrogenase reaction is rate-limiting in TPN-dependent oxidation. Since the oxidation rates of external and *iso*citrate-generated TPNH are both lower with cytochrome *c* as acceptor than with 2:6-dichlorophenol-indophenol, it would seem that a further rate-limiting step may occur between cytochrome *c* and the flavin at which 2:6-dichlorophenol-indophenol accepts electrons. However, since with molecular oxygen as acceptor, external TPNH is much more slowly oxidized than *iso*citrate-generated TPNH, it

may well be that separate oxidation pathways exist for externally and internally generated TPNH.

Wenner and associates have shown that intact Ehrlich ascites cells do not oxidize externally generated TPNH with molecular oxygen as terminal electron acceptor (Wenner, Hackney & Herbert, 1957; Wenner, Hackney & Moliterno, 1958; Wenner & Hackney, 1959; Wenner, 1959). From studies of the hexose monophosphate shunt pathway these authors concluded that pyruvate is the natural electron acceptor for TPNH oxidation in the tumour cell. The existence of a TPNH–cytochrome *c* reductase in the Novikoff hepatoma has been demonstrated by Birns, Essner & Novikoff (1959).

The rate of *iso*citrate oxidation with DPN as cofactor remained linear over 10 min. with 2:6-dichlorophenol-indophenol as electron acceptor, and over 22 min. with molecular oxygen as acceptor (Hawtrey & Silk, 1960*b*). However, with cytochrome *c* as acceptor, DPN-linked oxidation was strongly inhibited after an initial period of 2 min. (Fig. 1). This may be due to inhibition of further cytochrome *c* reduction by an accumulation of reduced cytochrome *c*. Alternatively, it may be due to reoxidation of reduced cytochrome *c* despite cyanide blocking of cytochrome oxidase. This could establish a steady level, in which reduction is balanced by oxidation.

The amount of coenzyme required to saturate both the DPN- and TPN-dependent *iso*citric dehydrogenases of Ehrlich mitochondria varied according to the electron acceptor used. Thus with 2:6-dichlorophenol-indophenol as acceptor, 1·35 mM-DPN afforded maximum enzyme activity (Fig. 2), but with molecular oxygen as acceptor only 0·24 mM-DPN was required to attain maximal respiration (Table 1). Similarly with TPN, a concentration of 0·3 mM was adequate to saturate the system with 2:6-dichlorophenol-indophenol as electron acceptor (Fig. 3), whereas only 75 μM-TPN was required to afford maximum activity with cytochrome *c* as terminal acceptor (Fig. 4).

The existence of separate DPN- and TPN-dependent *iso*citric dehydrogenases in Ehrlich mitochondria was corroborated by determination of pH–activity curves for the two enzymes in tris–acetate buffers with 2:6-dichlorophenol-indophenol as electron acceptor. The DPN-dependent enzyme exhibited a pH optimum of 7·1 with a fall in activity on either side of this value, whereas the TPN-dependent system showed a pH optimum at 6·2 with an inflexion at pH 7·8 (Fig. 5). Rat-liver lactic dehydrogenase exhibits similar properties (Navazio, Ernster & Ernster, 1957) in that the enzyme is both DPN- and TPN-dependent, with pH optima of 7·6 and 6·0 for the two cofactors respectively. The difference between the diaphorase activities of Ehrlich mitochondria in phosphate and tris–acetate buffers is not readily explicable, but is not without parallel (Silk, Hawtrey & Macintosh, 1958).

A further distinction between the DPN- and TPN-linked *iso*citric-dehydrogenase systems in Ehrlich mitochondria was established by aging experiments. Two-thirds of the DPN-diaphorase activity was lost on 84 hr. storage at 0°, whereas the TPN-linked system was little affected under the same conditions (Table 3). Plaut & Sung (1954) have reported that the DPN-dependent *iso*citric dehydrogenase of animal tissues is much more labile than the corresponding TPN-dependent enzyme. However, Williams-Ashman (1953) has observed a decrease in TPN-linked *iso*citric dehydrogenase activity on storage of a bicarbonate extract prepared from acetone-dried powders of whole Ehrlich ascites cells.

Phosphorylation is apparently coupled to electron transport during both DPN- and TPN-dependent *iso*citrate oxidation, but very low P:O ratios were observed for this process. The highest measured was 1·31 (Table 4). In rat-liver mitochondria and heart sarcosomes, phosphorylation occurs only after transfer of electrons from TPNH to DPN via a transhydrogenase (Kaplan *et al.* 1956), but in Ehrlich mitochondria, which do not contain a transhydrogenase, phosphorylation is associated with the direct TPNH to oxygen pathway (Table 4).

The similar inhibitory action of amytal on both DPN– and TPN–cytochrome *c*-reductase activities in Ehrlich mitochondria might indicate an effect on a common flavin (Table 5). The failure of amytal to affect DPN-linked diaphorase activity with 2:6-dichlorophenol-indophenol as electron acceptor is contrary to its effect on intact rat-liver mitochondria, where, at the same concentration, it is reported to block DPN-linked oxidations completely through an action on the electron-transfer chain between DPN and flavoprotein (Purvis, 1958*b*; Ernster, Jalling, Löw & Lindberg, 1955; Chance, 1956).

Chlorpromazine exerts a stimulating effect on TPN-dependent *iso*citrate oxidation in Ehrlich mitochondria, but has no effect on DPN-linked oxidation (Table 5). In rat-liver mitochondria, chlorpromazine inhibits DPN-linked oxidations by 50% when cytochrome *c* is used as electron acceptor (Dawkins, Judah & Rees, 1959).

The effect of 2:4-dinitrophenol on TPN–cytochrome *c*-reductase activity is in keeping with its action on this system isolated from bovine heart muscle (Lang & Nason, 1959), but its lack of effect on the DPN-dependent system is unexpected (Table 5).

The failure of antimycin A to inhibit TPN-dependent activity, except during an initial period of 2 min., is in contrast with its effect on most

normal-cell mitochondria, in which it acts as a potent blocker of electron transport (Chance, 1956). The unusual effect in Ehrlich mitochondria may be due to inactivation of the antimycin A. The TPN–cytochrome *c* reductase isolated from bovine heart muscle by Lang & Nason (1959) is, however, insensitive to antimycin A. The effects of dioxan on electron transport in Ehrlich mitochondria are the opposite of its effects on normal rat-liver mitochondria. Thus with 2:6-dichlorophenol-indophenol as electron acceptor, dioxan enhanced both DPN- and TPN-dependent *iso*citric-dehydrogenase activities in the tumour particulates (Fig 6), but depressed TPN-dependent diaphorase activity in rat-liver mitochondria, which do not contain a DPN-dependent enzyme (Table 7). The effect of dioxan apparently depends upon the electron acceptor used, since it depressed both the DPN- and TPN-linked *iso*citric-dehydrogenase activities of Ehrlich mitochondria, with either cytochrome *c* or molecular oxygen as terminal acceptor (Table 6, Fig. 7). In normal rat-liver mitochondria, dioxan caused a stimulation of *iso*citric-dehydrogenase activity in the presence of DPN + TPN, and also an increase in the oxidation rate of externally generated TPNH, although it depressed the oxidation rate of TPNH generated from *iso*citrate oxidation in the presence of TPN. The action of dioxan may be similar to that of acetone treatment, which Ernster (1958) has found to enhance the TPN-diaphorase activity of rat-liver mitochondria.

Digitonin has been used by Kaplan, Colowick & Neufeld (1953) to extract transhydrogenase activity in a non-sedimentable form from bovine heart mitochondria, whereas Stein, Kaplan & Ciotti (1959) have shown that digitonin causes an increase in the transhydrogenase activity of pigeon-liver mitochondria. Vignais & Vignais (1957) have reported similar effects of digitonin on reaction rates in mitochondria. The DPN-dependent *iso*citric-dehydrogenase activity of Ehrlich mitochondria was enhanced by digitonin treatment, which was without effect on the TPN-dependent diaphorase (Fig. 8).

The effects of digitonin, dioxan, amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on electron transport in Ehrlich mitochondria require further study with a variety of electron acceptors before the mechanism of *iso*citrate oxidation in these tumour particulates will be fully understood.

SUMMARY

1. Ehrlich ascites-cell mitochondria possess both diphosphopyridine nucleotide- and triphosphopyridine nucleotide-dependent *iso*citric dehydrogenases, in contrast with rat-liver mitochondria, which contain only the triphosphopyridine nucleotide-specific enzyme. Both dehydrogenase systems in Ehrlich mitochondria are linked to oxygen and the diphosphopyridine nucleotide-dependent enzyme is some three to four times as active as its triphosphopyridine nucleotide-specific counterpart.

2. Pyridine nucleotide transhydrogenase is completely absent from Ehrlich ascites-cell mitochondria.

3. With 2:6-dichlorophenol-indophenol and cytochrome *c* as electron acceptors, externally added reduced triphosphopyridine nucleotide is more rapidly oxidized than reduced triphosphopyridine nucleotide generated from *iso*citrate oxidation in the presence of triphosphopyridine nucleotide. With molecular oxygen as acceptor, externally added reduced triphosphopyridine nucleotide is oxidized more slowly than *iso*citrate-generated reduced triphosphopyridine nucleotide. Thus the dehydrogenase reaction appears to be rate-limiting in triphosphopyridine nucleotide-dependent *iso*citrate oxidation, whereas a further rate-limiting step may occur between cytochrome *c* and the flavin at which 2:6-dichlorophenol-indophenol accepts electrons.

4. The dependence of *iso*citric-dehydrogenase activity on coenzyme concentration has been determined with 2:6-dichlorophenol-indophenol, cytochrome *c* and molecular oxygen as electron acceptors.

5. Determination of pH–activity curves in tris buffers showed that diphosphopyridine nucleotide-dependent *iso*citric dehydrogenase has a pH optimum of 7·1 with a fall in activity on either side of this value, whereas the triphosphopyridine nucleotide-dependent system has a pH optimum of 6·2 with an inflexion at pH 7·8.

6. Mitochondria stored for 84 hr. at 0° lose two-thirds of their diphosphopyridine nucleotide-diaphorase activity, whereas triphosphopyridine nucleotide-diaphorase activity is unaffected.

7. Electron transfer resulting from both diphosphopyridine nucleotide and triphosphopyridine nucleotide-dependent *iso*citrate oxidation is coupled to phosphorylation, but it was not possible to obtain P:O ratios higher than 1·31.

8. The effects of digitonin, dioxan, amytal, chlorpromazine, 2:4-dinitrophenol and antimycin A on electron transport in Ehrlich ascites-cell mitochondria have been determined with 2:6-dichlorophenol-indophenol, cytochrome *c* or molecular oxygen as terminal acceptors. The effects have been contrasted with those on normal rat-liver mitochondria.

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A Determination of K_s, the Substrate Constant, for Serum Cholinesterase

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K_s, the substrate constant (Dixon & Webb, 1958), has not previously been determined for esterases. Slater (1955) has suggested that if the constant k_3, governing the rate of breakdown of the enzyme–substrate complex, can be varied independently of K_s then the plot of K_m against V_{max}, the maximum reaction velocity, will give K_s on extrapolation to the K_m axis. In this paper the alcohol-activation and reversible inhibition of the hydrolysis of certain nitrophenyl carboxylic esters by human serum cholinesterase have been employed to evaluate K_s by the use of the suggestions, but not the procedure, of Slater (1955).

Knowledge of K_s is particularly important when evaluating the substrate-specificity pattern of esterases since it is K_s, and not K_m, that is a

measure of the affinity of the enzyme for the substrate. Knowledge of K_s is also desirable when determining activation energies, since K_s is a true equilibrium constant whereas K_m includes a rate constant k_3 as well as the equilibrium constant K_s.

EXPERIMENTAL

Activities were measured in most instances with a Beckman DK-2 recording spectrophotometer equipped with a time drive and a temperature-regulated cell-holder. Usually, human serum was used as a source of cholinesterase. As observed by Aldridge (1953), human serum does not contain measurable B- or ali-esterase activity. The only esterases hydrolysing *o*-nitrophenyl butyrate, *p*-nitrophenyl butyrate and similar esters were therefore cholinesterase and A-esterases.

The DK-2 can be made to record the difference in extinction between the contents of a reference cell and an unknown cell as a function of time. The reference solution contained 0·1 mM-di*iso*propyl phosphorofluoridate (DFP) but otherwise was identical with the unknown solution. The DFP completely and quickly inhibited the cholinesterase, but left the A-esterases unaffected. The rate of change of extinction between the reference and unknown cells was therefore a measure of the cholinesterase activity. The A-esterase activity towards *o*-nitrophenyl butyrate was negligible (Main, Miles & Braid, 1961), but was of the same order as the cholinesterase activity towards *p*-nitrophenyl butyrate, *p*-nitrophenyl acetate and *o*-nitrophenyl acetate.

o-Nitrophenyl butyrate was prepared and purified according to the method of Main *et al.* (1961). *o*-Nitrophenyl acetate was purchased from the Eastman Kodak Co. (Rochester, N.Y., U.S.A.). *p*-Nitrophenyl acetate and *p*-nitrophenyl butyrate were prepared according to the method of Aldridge (1953). The *p*-nitrophenyl butyrate was purified in a manner similar to that used for *o*-nitrophenyl butyrate.

Stock solutions (0·5M) of these esters were prepared by weighing out the appropriate amount and dissolving in slightly acid methanol (e.g. 1·046 g. of *o*-nitrophenyl butyrate to 10 ml. with methanol).

Substrate solutions were prepared by pipetting appropriate volumes of stock solution into 50 mM-sodium phosphate buffers, pH 7·6. The solubility of *o*-nitrophenyl butyrate at 25° under these conditions was about 1·5 mM. A mM-*o*-nitrophenyl butyrate solution was prepared by pipetting 0·20 ml. of stock into 100 ml. of buffer. Lower concentrations were prepared by diluting the mM-*o*-nitrophenyl butyrate solution.

The alcohols were reagent-grade commercial products. Appropriate volumes were diluted with phosphate buffer to obtain the final alcohol concentration (v/v) desired.

Human serum was obtained from the blood of volunteers. No anticoagulants were used. To obtain comparable results it was important to use fresh serum which had not been frozen nor stored for periods longer than a week. The K_m with *o*-nitrophenyl butyrate decreased gradually from about 0·14 mM for fresh serum to values as low as 0·06 mM. This storage effect had no relevance to the effect of alcohols, which will be discussed. However, the results obtained from studies with alcohols and serum of differing ages suggested that the shift in K_m was associated with K_s. The possibility is suggested that a naturally occurring inhibitor is associated with cholinesterase in fresh serum and that this inhibitor gradually falls away during storage.

The serum was diluted with buffer containing the appropriate alcohol, 0·1% in acacia, so that the concentration of the alcohol in the enzyme solution was identical with that of the substrate solution. Typically 1·0 ml. of serum was diluted to 100 ml. with appropriate buffer and 1 ml. of this dilution containing 0·01 ml. of serum was used for an activity determination. The dilution was stored continuously in crushed ice.

The reference enzyme dilution was prepared by taking 25 ml. of the initial dilution and adding 0·10 ml. of a 25 mM-DFP solution made with propan-2-ol.

A typical determination was performed as follows: 1·0 ml. of a suitable serum dilution was pipetted into a clean, dry 25 ml. conical flask (A). Similarly, 1·0 ml. of the corresponding reference dilution (0·1 mM in DFP) was pipetted into flask (B). Buffered substrate (5·0 ml.) was pipetted simultaneously into each of the two flasks. After mixing, and as quickly as possible, a suitable amount of A was poured into the 'unknown' cell and a suitable amount of B into the 'reference' cell. The rate of change of extinction was plotted for at least 10 min. A straight line, whose slope was a measure of the activity, could usually be obtained over the major part of this interval for each substrate concentration with suitable variations of enzyme concentration. Just before every determination, the ratio-recording spectrophotometer was calibrated with respect to 0 and 100% transmittance with a freshly prepared reaction mixture corresponding to the final contents of flask B.

The extinction of *o*-nitrophenol was measured at 414 mμ (Main *et al.* 1961) and that of *p*-nitrophenol at 400 mμ.

The temperature was controlled by circulating the water from a temperature-controlled bath through the cell-holder. The constant-temperature bath was kept at 24·5° to maintain the thermometer in the regulator at 25·1°.

The activity was calculated in terms of μmoles of nitrophenol liberated/ml. of serum/min.

Occasionally activities were determined with the Hilger Uvispek spectrophotometer as follows. Buffered substrate (5·0 ml.) was pipetted into two or more volumetric flasks A and B. To B was added 0·05 ml. of 25 mM-DFP. The flasks were placed in a bath at 25° and 0·2–1·0 ml. of enzyme dilution containing a suitable amount of serum (0·004–0·05 ml., depending on the substrate and alcohol concentration) was pipetted into both flasks A and B and the time was noted. After a suitable digestion time (10–30 min., again depending on conditions) 0·05 ml. of DFP was added to flask A. Flasks A and B were then immediately made up to the 10 ml. mark with 40% (v/v) ethanol solution. The ethanol terminated the A-esterase activity. The colour was stable for at least 1 hr. and could be read at any convenient time on the Hilger Uvispek instrument.

RESULTS

The activities at five different concentrations of *o*-nitrophenyl butyrate were determined in 0, 0·5, 1, 2·5 and 3·5% butanol. The results were plotted according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. As shown in Fig. 1, the extrapolations of the experimental

points were carried below the $1/s$ axis into the lower left-hand quadrant, where the system of lines obtained approximated a common point of intersection (P). Interpretation of the point P led to a new method for evaluating K_s.

Each line corresponded to the activities at a single butanol concentration. The intercept on the vertical axis gave the reciprocal of V, the maximum velocity. The intercept on the horizontal axis gave the negative reciprocal of K_m, where K_m has the Briggs & Haldane (1925) meaning:

$$K_m = (k_2+k_3)/k_1 = K_s+k_3/k_1. \qquad (1)$$

The individual rate constants are those used to describe the reaction

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+\text{products},$$

where E is the enzyme, S is the substrate and ES is the enzyme–substrate complex.

The point P represents the average $-1/s$ values of the points of intersection. If a line is drawn from P perpendicular to the $1/s$ axis, the intercept is equal to $-1/K_s$.

This conclusion was reached by considering the limiting value of $-1/K_m$ when $1/V$ is equal to infinity.

If P is joined to the $1/v$ axis at infinity, the line will be parallel to the $1/v$ axis since parallel lines meet at infinity.

On the $1/v$ axis, $1/s = 0$, and by substituting into

$$\frac{1}{v} = \frac{K_m}{V}.\frac{1}{s}+\frac{1}{V} \qquad (2)$$

then $1/v = 1/V$ and, at infinity, $V = 0$. Similarly, on the $1/s$ axis, $1/s = -1/K_m$. The intercept on the $1/s$ axis of the line joining P to infinity on the $1/v$ axis is therefore $-1/K_m$. But $V = 0$ for this line, and since e, the enzyme concentration, is constant, it follows from the relationship

$$V = k_3.e \qquad (3)$$

that $k_3 = 0$. Substituting into (1), $K_m = K_s$ and $-1/K_m = -1/K_s$. Similarly it can be shown that the intercept on the $1/v$ axis of the line joining P to $1/s$ at infinity is $-1/k_2.e$. The slope of the line joining P to the origin is then $1/k_1.e$.

The five lines of Fig. 1 intersect at ten different points. The deviation from a common point of intersection appears to be random, suggesting the limits of experimental precision, rather than a progressive shift in K_s with increasing butanol concentration. Summing the values at each intersection and dividing by 10 gave K_s 0·072 ± 0·026 mM. But this method did not appear to be entirely satisfactory since equal weight was given to the value at each intersection regardless of the angle of intersection. The precision appears to depend on the angle of intersection, with the wider angle giving the higher precision. This was shown when K_s values were grouped in descending order of intersecting angles as in Table 1.

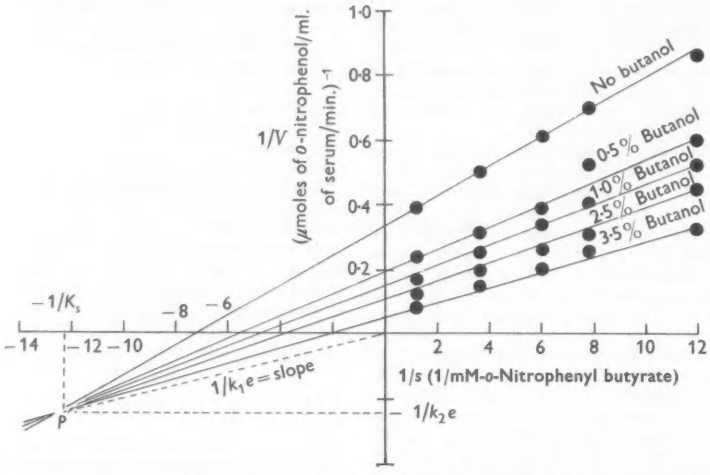

Fig. 1. k_3-Activation plot. The Lineweaver & Burk (1934) plot of reciprocal activities against corresponding reciprocal *o*-nitrophenyl butyrate concentrations. Each line is the plot at the given butanol concentration. Intercepts on the $1/s$-activity axis give $1/V$ and on the 1/mM-*o*-nitrophenyl butyrate axis, $-1/K_m$. The values of these intercepts obtained from a plot on 16 in. × 10 in. graph paper are given in Table 2.

It is evident that more confidence may be placed in the K_s values obtained at the wider angles of intersection. These observations suggest that the precision of the method may be somewhat greater than the standard deviation $\pm 0{\cdot}026$ mM implies.

K_s may also be evaluated by plotting V against K_m by the method of Slater (1955). Each butanol concentration was associated with characteristic and particular values of K_m and V. These were calculated from the intercepts on the $1/v$ and $1/s$ axis as suggested by Fig. 1. In practice K_m and V were obtained from a plot on 10 in. × 16 in. graph paper and are given in Table 2.

If the plot of V against K_m gives a straight line, then according to Slater's (1955) equation

$$K_m = V\frac{1}{k_1 e} + K_s \qquad (4)$$

K_m will equal K_s when V is equal to 0. As shown in Fig. 2, the plot of V versus K_m did approximate a straight line, from which K_s could be evaluated.

Evaluation of K_s from the point of intersection (P), Fig. 1, has advantages over the use of Slater's (1955) K_m versus V plot. Evaluation from the point of intersection is more direct, making use of primary activity data, whereas the Slater (1955) method uses secondary data. The intersection method also suggests the experimental conditions which will give the most precise K_s evaluation, whereas this is not readily apparent from the plot of K_m against V.

Both V and K_m increased with increasing butanol concentration (Table 2). The increase in V with butanol concentration approximated a positive linear function, as shown in Fig. 3. The relationship could be expressed by the equation $V = V_0 + kB$, where V is the maximum velocity at butanol concentration B, V_0 is the maximum velocity in water and k is the slope of the line.

According to (3) V is a function of k_3, the rate constant governing the breakdown of the enzyme–substrate complex. Since the enzyme concentration was constant, the increase in V with increased butanol concentration suggested that butanol was increasing k_3.

Table 1. *K_s values grouped in descending order of intersecting angles*

No. of points of intersection	Average angle of intersection (degrees)	$K_s \pm$ deviation (mM)
1	14·2	0·072
2	8·3 ± 2·3	0·069 ± 0·003
3	5·5 ± 2·0	0·073 ± 0·021
4	3·4 ± 2·1	0·081 ± 0·040

Table 2. *Effect of butanol on the o-nitrophenyl butyrate K_m and V of human serum cholinesterase*

K_m and V were obtained by extrapolation according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. For each butanol concentration the activities were determined at 1, 0·33, 0·2, 0·15 and 0·1 mM-*o*-nitrophenyl butyrate concentrations, with 1·0 ml. of diluted serum containing 0·01 ml. of the pooled serum from four individuals.

Butanol (%, v/v)	$V_{max.}$ (μmoles of *o*-nitrophenol/ml. of serum/min.)	K_m (mM)
0	3·01	0·139
0·5	5·18	0·173
1·0	7·64	0·250
2·5	11·1	0·318
3·5	18·2	0·455

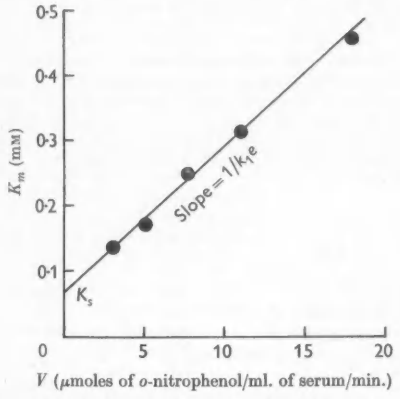

Fig. 2. Plot of V against K_m with extrapolation to the K_m axis giving K_s. (For conditions see Table 2.)

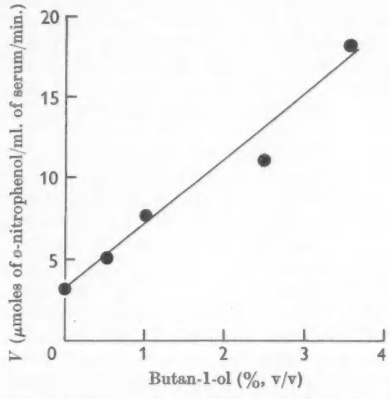

Fig. 3. Effect of butanol on V, showing the linearity of the relationship. (For conditions see Table 2.)

Table 3. *Effect of butanol on the* p*-nitrophenyl butyrate K_m and V of human serum cholinesterase*

K_m and V were obtained by extrapolation according to the Dixon (1953) modification of the Lineweaver & Burk (1934) plot. The activities were determined with the Beckman DK-2 recording spectrophotometer at 1, 0·33 and 0·2 mM-*p*-nitrophenyl butyrate concentrations in duplicate for each butanol concentration. With water, 0·02 ml. of the pooled serum from four individuals was used for each determination but this was increased to 0·05 ml. for the activities determined in butanol.

Butanol (%, v/v)	V (μmoles of *p*-nitrophenol/ml. of serum/min.)	K_m (mM)
0	1·06	0·80
0·2	0·83	0·72
0·5	0·63	0·63
1·0	0·40	0·50

Table 4. *Effect of alcohol chain-length on the activation of* o*-nitrophenyl butyrate hydrolysis by human serum cholinesterase*

Substrate solutions were 0·20 M with respect to each alcohol. Activities were determined at pH 7·6 and 25° in the DK-2 recording spectrophotometer. K_m and V were determined with 1, 0·33 and 0·2 mM-*o*-nitrophenyl butyrate concentrations and 0·010 ml. of serum from four individuals in a suitable dilution.

	$V_{max.}$ (μmoles of *o*-nitrophenol/ml. of serum/min.)	K_m (mM)
Water	3·28	0·135
Methanol (0·74 M)	3·60	0·141
Ethanol	4·00	0·151
Propan-1-ol	5·88	0·202
Butan-1-ol	8·50	0·244

Activation of enzyme-catalysed ester hydrolysis by alcohols is not common, but inhibition by alcohols has been widely observed (e.g. Huggins & Lapides, 1947). It was therefore of interest to determine whether alcohol inhibition of enzyme-catalysed ester hydrolysis was also a k_2-dependent effect.

Butanol inhibited the hydrolysis of *p*-nitrophenyl butyrate by cholinesterase. Both K_m and V decreased, as shown in Table 3. Competitive inhibition typically results in an increased K_m. With non-competitive inhibition K_m remains unchanged whereas V decreases with decreasing effective enzyme concentration, k_3 remaining unchanged. Mixed competitive–non-competitive inhibition results in increased K_m and decreased V. The butanol inhibition of *p*-nitrophenyl butyrate hydrolysis by cholinesterase where both V and K_m decrease is therefore unique and characteristic of k_3 inhibition. The Lineweaver–Burk plots approximated a common point of intersection when extrapolated to the lower left-hand quadrant, from which the *p*-nitrophenyl butyrate K_s was 0·38 mM. Butanol inhibition of cholinesterase *p*-nitrophenyl butyrate hydrolysis appeared therefore to be a k_2-dependent effect similar to *o*-nitrophenyl butyrate activation, but in reverse. A comparison of the K_s for *o*-nitrophenyl butyrate with the K_s for *p*-nitrophenyl butyrate suggested that the affinity of serum cholinesterase for *o*-nitrophenyl butyrate was five times as great as for *p*-nitrophenyl butyrate.

The effect of butanol on the hydrolysis of *o*-nitrophenyl acetate and *p*-nitrophenyl acetate catalysed by human serum cholinesterase was also examined. With *o*-nitrophenyl acetate an activation similar to that with *o*-nitrophenyl butyrate was observed, whereas with *p*-nitrophenyl acetate an inhibition corresponding to that of *p*-nitrophenyl butyrate occurred.

The effect of butanol on the hydrolysis of *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate by a Strelitz (1944) Stage-2 purified horse-serum cholinesterase preparation was also investigated briefly. Butanol activated the *o*-nitrophenyl butyrate hydrolysis strongly. However, the *p*-nitrophenyl acetate hydrolysis results were difficult to interpret because the *p*-nitrophenyl acetate K_m was in the order of 60 mM whereas the activities were determined at substrate concentrations of 1 mM and less. The accuracy of K_m and V determined by extrapolation under these conditions is necessarily too low to make measurement of differences meaningful. This impasse may be

Table 5. *Effect of the isomeric configuration of butanol and of acetone on the activation of* o*-nitrophenyl butyrate hydrolysis by human serum cholinesterase*

Activities were determined with mM-*o*-nitrophenyl butyrate in 5% (v/v) solutions of butanol at pH 7·4, 25°; 0·20 ml. of a 1:20 serum dilution, equivalent to 0·01 ml. of serum from one individual, was added to 5 ml. of buffered substrate containing the butanol, according to the method described in the Experimental section with the Hilger Uvispek.

Isomer	Activity (μmoles of *o*-nitrophenol/ml. of serum/min.)	Percentage of control
Control (no butanol)	2·82	100
Butan-1-ol	8·45	298
2-Methylpropan-1-ol	4·81	171
2-Methylpropan-2-ol	4·64	164
Butan-2-ol	3·15	112
Butyric acid	2·90	103
Acetone*	4·26	151

* 3% (v/v) Acetone by DK-2 ratio-recording spectrophotometer.

overcome by using higher alcohol concentrations, which will permit the use of higher substrate concentrations.

Investigation of the effect of other alcohols on the hydrolysis of *o*-nitrophenyl butyrate showed that each alcohol activated. At equivalent concentrations, the activation increased with increasing chain length (Table 4). A common point of intersection was approximated when the activities were plotted by the method used in Fig. 1. The K_s value calculated was $0{\cdot}077 \pm 0{\cdot}010$ mM and was thus within the experimental limits of the K_s value obtained by varying the butanol concentration ($0{\cdot}072 \pm 0{\cdot}026$ mM).

Pentan-1-ol was also tested and activated more than butanol at equivalent concentrations. These observations suggested that the activating effect of alcohols is through k_3.

The effect of isomeric configuration and of acetone is given in Table 5. Butan-1-ol activated more than its isomers, but all isomers and acetone did activate. Butyrate ion did not activate.

DISCUSSION

The results described have been interpreted to suggest that K_s was constant whereas k_3 varied. Variation in k_3 has been inferred from the experimentally observed simultaneous increase or decrease in K_m and V. The approximation to a common point of intersection of the Lineweaver–Burk plots suggested that K_s was constant.

The case of simultaneous competitive inhibition and k_3 activation or inhibition has been investigated by M. Dixon & E. C. Webb (personal communication). Analysis of their hypothesis by the author suggested that K_m would not be a linear function of V should simultaneous variation of K_s and k_3 occur. The plot of K_m against V in Fig. 2 would be a curved line corresponding to a progressive shift in the lines of intersection. The observation that different alcohols yielded a similar K_s value was consistent with the interpretation that simultaneous competitive inhibition and k_3 activation had not occurred. This followed from the likely assumption that different alcohols would have different hypothetical K_i values.

There is at least one hypothetical case where both K_s and k_3 could vary simultaneously and give a straight-line plot of K_m against V. This line would pass through the origin, implying that k_2 is 0. But this would not necessarily be true since the plot would be obtained by a simultaneous variation of K_s (k_2/k_1) with k_3. The Lineweaver–Burk plots for this would result in a series of parallel straight lines. Since no point of intersection would result no K_s could be derived.

The characteristics of the activation and inhibition described in this work are not readily interpreted by the usual concepts of specific interaction between the enzyme active centre and the activator or inhibitor.

The variety of solvents which activated, ranging from methanol to acetone, does not suggest a particular affinity for the active centre.

If it is postulated that butanol might have an affinity for the active centre on the basis of its similarity to the substrate, it must also be remembered that butyrate ion had no effect. Moreover butanol has a dual role depending on the substrate, activating *o*-nitrophenyl butyrate hydrolysis and inhibiting *p*-nitrophenyl butyrate hydrolysis. Finally when it is remembered that other quite different alcohols and acetone also activated, the concept of direct interaction appears more unlikely.

This is not to say that these solvents do not exert their influence in the region of the active centre. The enzyme protein is normally surrounded by water, which plays two roles during hydrolysis. It is the supporting medium and it is a reactant. It is suggested that the properties of this environment affect k_3, governing the rate at which the enzyme–substrate complex breaks down. Addition of solvents displaces water and thus may alter the properties of the environment on which k_3 is dependent.

This k_3 effect may be termed an 'environmental' effect to distinguish it from the specific-interaction effects which are characteristic of competitive and non-competitive inhibitions and activation.

The property of the environment which the alcohols change and on which k_3 is dependent is not known. However, it seems possible that the dielectric constant may be concerned. Laidler (1955) has stated that: 'A reaction in which the activated complex is more polar than the reactants will be accelerated by an increase in the dielectric constant; conversely one in which the complex is less polar will be slowed down'. Addition of alcohols to water decreases the dielectric strength, and the decrease is proportional to the chain length of the alcohol. However, at the alcohol concentrations used, the change in dielectric strength does not appear to be sufficiently large to account for activating and inhibiting effects. The activation of *ortho*-orientated nitrophenol esters and the inhibition of *para*-orientated nitrophenol esters may be related to the relative polarity of these compounds. *ortho*-Compounds may form rings with adjacent groups, thus making them less polar than *para*-compounds.

The experimental method devised by Slater depended on varying the rate of breakdown of the enzyme–substrate complex into products by varying the concentration of a second substrate

necessary for this breakdown. Consequently hydrolytic enzymes could not be studied since the reactions could not be carried out in the absence of water. It seems most unlikely that the effect of alcohols is in any way related to the conception of a second substrate. Consequently Slater's (1955) experimental treatment is related to the present procedure only by the fact that both methods depend on varying k_3 independently of K_s. Slater's (1955) method appears to apply only when k_3 is a first-order reaction constant, dependent only on the concentration of ES. This condition is satisfied in the present procedure since butanol (etc.) evidently does not act as a second substrate, nor does it appear to interact directly with the active centre.

If the reversible effect of alcohols on other hydrolytic enzymes is the same as that for cholinesterase, then this effect may provide a useful means of determining the K_s for these enzymes. When the concentration of the enzyme is also known, then the individual rate constants k_1, k_2 and k_3 may also be calculated. The relative magnitudes of the individual reaction constants can be estimated and this information may provide valuable insights into the reaction mechanism. It seems likely that alcohols will also affect K_s, but evidently the effect on k_3 is much greater than the effect they might have on K_s.

SUMMARY

1. The hydrolysis of *o*-nitrophenyl butyrate by human serum cholinesterase was activated by alcohols and acetone.

2. Lineweaver–Burk plots of the activities at a number of butanol concentrations gave lines, which, on extrapolation into the lower left-hand quadrant, approximated a common point of intersection. The point of intersection could be used to determine K_s, k_2e and k_1e directly from the plot. It is suggested that this plot is typical of k_3 activation.

3. The activation was dependent on the chain length, methanol activating least and pentan-1-ol most. Butan-1-ol activated more than its stereoisomers.

4. The hydrolysis of *p*-nitrophenyl butyrate was inhibited by butan-1-ol. Lineweaver–Burk plots of the activities at a number of butan-1-ol concentrations approximated a common point of intersection in the lower left-hand quadrant in a manner similar to the activation plot. This plot is probably typical of k_3 inhibition.

5. The human serum cholinesterase *o*-nitrophenyl butyrate K_s was 0·072 mM, compared with K_m 0·139 mM, and the *p*-nitrophenyl butyrate K_s was 0·38 mM, compared with K_m 0·80 mM. The serum cholinesterase K_m of these substrates has therefore a mixed meaning, being both k_3- and K_s-dependent.

6. *o*-Nitrophenyl butyrate hydrolysis by a partially purified horse-serum cholinesterase preparation was also activated by butan-1-ol.

7. *o*-Nitrophenyl acetate hydrolysis by human serum cholinesterase was activated by butan-1-ol and *p*-nitrophenyl acetate hydrolysis was inhibited.

8. It is suggested that alcohols affect the activity of hydrolytic enzymes by changing some aspect of the environment on which k_3 is dependent. The effect has been called 'environmental' to distinguish it from specific interaction. On the basis of an environmental effect alcohols may provide a useful method for determining the K_s of many hydrolytic enzymes.

The author is particularly grateful to Dr M. Dixon and Dr E. C. Webb for their interest and valuable criticism of the work. He also takes pleasure in acknowledging the expert technical assistance of Mrs Kathleen Miles. In addition, thanks are gratefully extended to Dr P. E. Braid for his interest and patience during our numerous conversations.

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The Enzymic Synthesis of Aryl Sulphamates

2. THE EFFECT OF 3β-METHOXYANDROST-5-EN-17-ONE ON ARYLAMINE SULPHOKINASE*

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In the first paper of this series (Roy, 1960) it was shown that an enzyme system in mammalian livers could synthesize 2-naphthyl sulphamate, and related aryl sulphamates, by a series of reactions terminating in the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to the arylamine, this step being catalysed by a specific sulphokinase, arylamine sulphokinase. The rate of synthesis of 2-naphthyl sulphamate by such enzyme preparations from rat liver was greatly increased by the addition to the system of low concentrations of 17-oxo steroids. There was no comparable activation of preparations from guinea-pig liver, which were, on the contrary, strongly inhibited by 17-oxo steroids. It was suggested that the activation depended upon the formation of a steroid 17-enol sulphate, through the action of an unidentified sulphokinase, followed by the transfer of sulphate therefrom to 2-naphthylamine by a reaction which did not involve arylamine sulphokinase and which re-formed the 17-oxo steroid. The truly catalytic action of the steroid was obvious from the quantitative results: in a typical experiment (Roy, 1960) the addition of 50 μm-moles of *epi*androsterone to the reaction mixture gave an increase of 250 μm-moles in the amount of sulphamate formed.

The present paper deals with the kinetics of the activated reaction. In this work the rather insensitive assay used previously has been replaced by one based on the methylene-blue technique of Roy (1956), which is more sensitive but cannot be used with those steroids which themselves give rise to sulphate esters. The most suitable type of steroid for the investigation would have been an otherwise unsubstituted 17-oxo steroid such as androstan-17-one, but such compounds were not readily accessible in the required amounts and the compound chosen for detailed study was 3β-methoxyandrost-5-en-17-one, in which the methoxyl group effectively prevented any changes at the 3 position. Under the experimental conditions used it is extremely unlikely that any change could occur elsewhere, except perhaps at position 17.

* Part 1: Roy (1960).

As the action of the 17-oxo steroid is known to occur at some stage of the reaction subsequent to the formation of adenosine 3′-phosphate 5′-sulphatophosphate (Roy, 1960), it might be argued that it would have been preferable to study only the sulphokinase stage of the reaction with adenosine 3′-phosphate 5′-sulphatophosphate as the sulphate donor. However, as this latter reaction is the rate-limiting step in sulphamate synthesis (Roy, 1960) little would in fact have been gained by this procedure, and in this work the endogenous sulphate-activating enzymes of liver have been used to provide the adenosine 3′-phosphate 5′-sulphatophosphate. It must be stressed, however, that the kinetics of the formation of 2-naphthyl sulphamate which are described below are essentially the kinetics of the sulphokinase reaction.

EXPERIMENTAL

Preparation of the enzyme. The enzyme system was prepared as previously described (Roy, 1960) from the supernatant obtained by centrifuging for 2 hr. at 20 000*g* a 25% homogenate of rat or guinea-pig liver in 0·15M-KCl–1 mM-ethylenediaminetetra-acetate, pH 7. The active fraction was precipitated between 1·5 and 2·3M-$(NH_4)_2SO_4$ and was dissolved in water so that 1 ml. of the solution corresponded to 500 mg. of liver. Preparations from rat liver could be stored frozen for considerable periods without loss of activity but those from guinea-pig liver were considerably less stable and often became inactive on storage for 12 hr.

The above preparation (the complete enzyme system) contained the enzymes responsible for the synthesis of adenosine 3′-phosphate 5′-sulphatophosphate from SO_4^{2-} ions and adenosine triphosphate (ATP) as well as several sulphokinases. The sulphokinase fraction was obtained as previously described (Roy, 1960) by inactivation of the adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing enzymes through removal of the protecting SO_4^{2-} ions by dialysis.

Preparation of adenosine 3′-phosphate 5′-sulphatophosphate. A crude preparation of this compound was made exactly as described by Roy (1960).

Assay of 2-naphthyl sulphamate synthesis. For the assay of the synthesis from ATP and SO_4^{2-} ions the reaction mixture had the following composition: 0·1 ml. of 0·2M-potassium phosphate buffer, pH 7·5; 0·1 ml. of 0·04M-$MgSO_4$–0·06M-K_2SO_4; 0·2 ml. of 0·02M-ATP (crystalline

sodium salt, Sigma Chemical Co.; adjusted to pH 7·5 with N-NaOH); 0·2 ml. of 0·01 M-2-naphthylamine hydrochloride; 0·2 ml. of aqueous 1% ethanol, or a solution therein of 3β-methoxyandrost-5-en-17-one (see below), and 0·2 ml. of the complete enzyme system diluted to correspond to 50–75 mg. of rat liver or 30–40 mg. of guinea-pig liver.

For the assay of the synthesis from adenosine 3′-phosphate 5′-sulphatophosphate the reaction mixture differed from the above in that the $MgSO_4$–K_2SO_4 and ATP solutions were replaced by 0·1 ml. of 0·02 M-magnesium acetate and 0·2 ml. of adenosine 3′-phosphate 5′-sulphatophosphate solution respectively. The complete enzyme system was replaced by 0·2 ml. of the sulphokinase fraction.

It should be noted that in these experiments phosphate buffer, pH 7·5, was used in place of the 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer, pH 8·2, used previously (Roy, 1960). This change was made as it was initially planned to link this work to an investigation requiring determination of the 17-oxo steroid; in such determinations phosphate buffers caused less interference than did tris buffers. In phosphate the pH curve of the reaction was rather flat with an optimum at about pH 7·5.

The solution of 3β-methoxyandrost-5-en-17-one was prepared immediately before use by dilution of 0·05 ml. of a 5 mM solution of the steroid in ethanol to 5 ml. with water: the final concentrations in the reaction mixture of the steroid and of ethanol were therefore 10 μM and 0·2% respectively. At this concentration ethanol was without effect on the reaction. If higher concentrations of steroid were required then the concentration of the stock ethanol solution was adjusted to keep constant the amount of ethanol in the reaction mixture.

After incubation for 2 hr. at 37° the reaction was stopped by the addition of 1 ml. of methylene blue reagent (Roy, 1956), and 5 ml. of $CHCl_3$ was added. The mixture was shaken vigorously for 1 min. and then centrifuged to break the emulsion. The aqueous layer was sucked off and a sample of the $CHCl_3$ layer, containing the methylene blue salt of the sulphamate, was dried over Na_2SO_4. The extinction of the $CHCl_3$ solution at 650 mμ was determined in a Unicam SP. 600 spectrophotometer against a suitable blank. This was normally the $CHCl_3$ extract prepared from a reaction mixture in which the enzyme had been added to the remaining constituents only immediately before the methylene blue. The amount of 2-naphthyl sulphamate present was calculated from a suitable calibration curve. The recovery of 2-naphthyl sulphamate added to the reaction mixture was 90%.

In the following sections the amount of sulphamate formed in 2 hr. under the above general conditions in the absence of any added steroid is denoted v_0, and that formed in the presence of 17-oxo steroid is denoted v.

RESULTS

Typical progress curves for the synthesis of 2-naphthyl sulphamate by the complete enzyme system from rat liver are shown in Fig. 1: they are similar to those previously obtained in the syntheses of other types of sulphate ester (Segal, 1955; Roy, 1956), as there is an initial lag phase followed by a linear phase in which the reaction is in the steady state and the velocity remains constant for at least 2 hr. The general shape of this curve is unaltered by the addition of 17-oxo steroid, although the lag phase is shortened (Fig. 1). This is as expected because of the greater activity of the arylamine sulphokinase in the presence of the steroid: as Segal (1955) pointed out, the length of the lag phase is inversely related to the velocity of the sulphokinase reaction. With enzyme preparations from guinea-pig liver the general shapes of the progress curves were similar to those shown in Fig. 1 except for the strong inhibition of the reaction by the 17-oxo steroid.

The effects of changes in the concentration of the enzyme system from rat liver are shown in Fig. 2. In the complete enzyme system, both in the presence of and in the absence of 17-oxo steroid, there is a critical enzyme concentration below which no activity can be detected. This effect is due to the prolongation of the lag phase at low enzyme concentrations and it is not apparent in the sulphokinase-catalysed reaction, as is also shown in Fig. 2. Presumably at low concentrations the activity of the sulphate-activating enzymes is so small that the amounts of adenosine 3′-phosphate 5′-sulphatophosphate produced are too low to allow the sulphokinase reaction to proceed at a measurable rate. The behaviour of the guinea-pig enzyme was exactly similar to that of the rat enzyme, apart from the inhibition by the 17-oxo steroid.

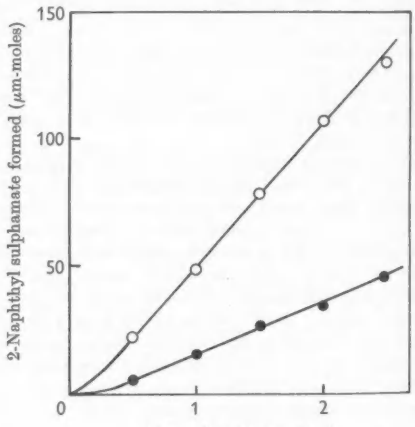

Fig. 1. Progress curves for the synthesis of 2-naphthyl sulphamate by rat-liver preparations. Volume of reaction mixture, 1 ml.; pH 7·5 in 0·02 M-phosphate buffer; SO_4^{2-} ion, 10 mM; Mg^{2+} ion, 4 mM; ATP, 4 mM; 2-naphthylamine, 2 mM. Control assays, ●; assays in 10 μM-3β-methoxyandrost-5-en-17-one, ○.

Effect of varying substrate concentrations. Fig. 3 shows the effects of changes in 2-naphthylamine concentration on the rate of sulphamate formation. With the rat enzyme there is a linear relationship between the substrate concentration and the reaction velocity, implying either that the substrate does not combine directly with the enzyme or, more probably, that the highest substrate concentration attainable, 2 mM, is still considerably less than the K_m for 2-naphthylamine. Whichever explanation is correct, values for K_m and maximum velocity V obviously cannot be obtained. In the

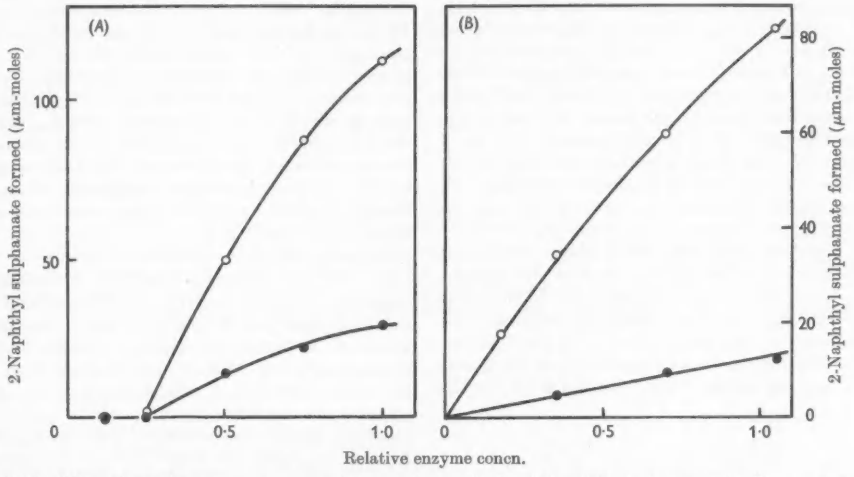

Fig. 2. Effect of varying enzyme concentrations on the synthesis of 2-naphthyl sulphamate by the complete enzyme system (*A*) and the sulphokinase fraction (*B*) from rat liver. In *A* the conditions were as in Fig. 1 except that the time of incubation was held at 2 hr.; in *B* the conditions were similar except that no sulphate was added and the ATP was replaced by 0·2 ml. of a standard preparation of adenosine 3′-phosphate 5′-sulphatophosphate (see text). A relative concentration of 1 corresponds to the amount of enzyme isolated from 50 mg./fresh wt. of liver. Control assays, ●; assays in 10 μM-3β-methoxyandrost-5-en-17-one, ○.

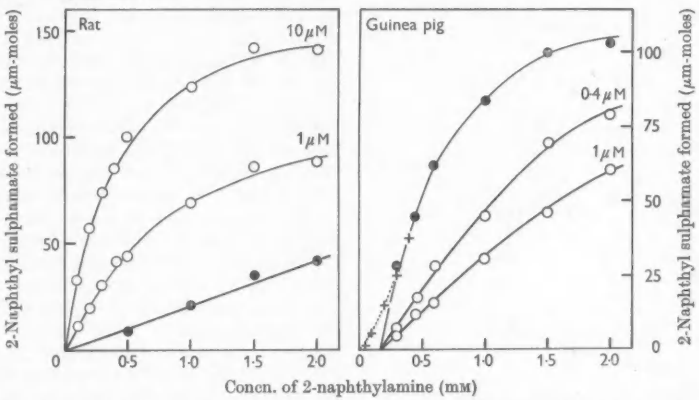

Fig. 3. Effect of varying substrate concentrations on the rate of 2-naphthyl sulphamate synthesis. Conditions were as in Fig. 1 except that the time of incubation was 2 hr. Control assays, ●; assays in the presence of the concentrations of 3β-methoxyandrost-5-ene-17-one indicated in the Figure, ○. The points + were calculated from assays containing double the usual enzyme concentration incubated for 4 hr. in order to obtain accurate values at low concentrations of substrate.

activated reaction, on the other hand, the effect of changes in 2-naphthylamine concentration is that characteristic of an enzyme reaction in which the substrate combines directly with the enzyme.

The corresponding curves for the guinea-pig enzyme are also shown in Fig. 3. With or without the addition of 17-oxo steroid they are typical of a reaction in which the substrate combines directly with the enzyme, but they are peculiar in that there is an apparent critical substrate concentration of between 0·1 and 0·2 mM-2-naphthylamine, below which no enzyme activity could be detected under the usual conditions of the assay. By using high concentrations of enzyme and prolonged times of incubation it was possible to show that the reaction did proceed at low concentrations of substrate but in this region the substrate curve of the reaction was sigmoidal in shape, as shown in Fig. 3.

In Fig. 4 are shown the usual reciprocal plots of $1/v$ against $1/s$ for the rat and of $1/v$ against $1/(s-x)$, where x is the apparent critical substrate concentration for the guinea-pig enzyme. The significance of the latter plot is discussed below. With the enzymes from both species the plots were linear and the effect of the 17-oxo steroid was the same in both: K_m was altered, as shown by the change in the intercepts on the $1/s$ axis, but V was unaltered, as shown by the constant intercept on the $1/v$ axis. The inhibition of guinea-pig arylamine sulphokinase by 3β-methoxyandrost-5-en-17-one is therefore of the competitive type, and from the graphs shown in Fig. 3 values of 0·5 mM-2-naphthylamine and 0·2 μM-3β-methoxyandrost-5-en-17-one can be calculated for K_m and K_i respectively, assuming for the latter that the inhibition is purely competitive. Similarly, the activation of rat arylamine sulphokinase must be of a 'competitive' type as the effect of the steroid is on K_m, but no value for this nor for V of the unactivated reaction can be obtained. As shown in Fig. 4, however, V for the activated reaction apparently does not change significantly with varying concentrations of steroid. In fact, slight alterations in V with changes in steroid concentration do occur but these are small in comparison with the corresponding changes in the apparent K_m. If it be assumed that the intercepts on the ordinate and abscissa of Fig. 4 are measures of V and K_m respectively, then an increase in the steroid concentration from 1 μM to 10 μM increases V by approximately 10%, whereas the apparent affinity of the enzyme for 2-naphthylamine, as measured by the apparent K_m, is increased by some 200%. It therefore seems justifiable, as a first approximation, to treat the activation as being of a purely competitive nature.

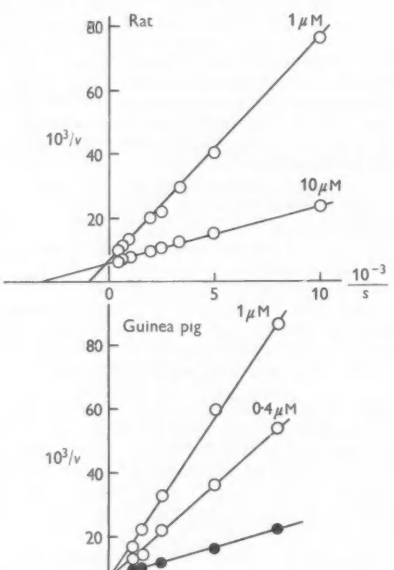

Fig. 4. Reciprocal plots corresponding to experiments similar to those shown in Fig. 3. With the guinea-pig enzyme, x is the apparent critical substrate concentration (see text).

Effect of varying concentrations of 17*-oxo steroid.* In Fig. 5 is shown the effect of varying concentrations of 3β-methoxyandrost-5-en-17-one on the reaction velocity. With rat arylamine sulphokinase the pronounced activation is obvious: this reaches a maximum at 10 μM-oxo steroid and decreases slightly at higher concentrations. Also shown in Fig. 5 are the comparable results for guinea-pig arylamine sulphokinase: the strong inhibition at concentrations of 1 μM-oxo steroid is obvious. As pointed out above, the inhibition is of the competitive type, but the results shown in Fig. 5 indicate that it is not purely competitive but partially competitive (Dixon & Webb, 1958), as the plot of $1/v$ against i is non-linear. This means that the true value for K_i must be less than the value of 0·2 μM-3β-methoxyandrost-5-en-17-one given above: as pointed out by Dixon & Webb (1958), the determination of K_i in cases of partial competitive inhibition is difficult and the method they suggest is inapplicable in the present case because of the limited solubility of 2-naphthylamine.

For both rat and guinea-pig arylamine sulphokinases the reciprocal plots of $1/v$ against $1/i$ (where i is the concentration of 17-oxo steroid whether it is an inhibitor or an activator) are non-linear. However, as shown in Fig. 6, the plots of

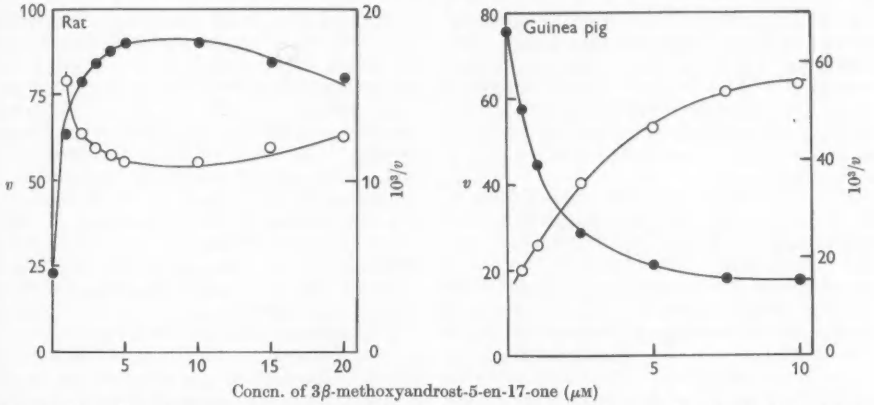

Fig. 5. Effect of varying concentrations of 3β-methoxyandrost-5-en-17-one on the rate of synthesis of 2-naphthyl sulphamate under the same general conditions as in Fig. 1 except that the time of incubation was 2 hr. and the substrate concentrations were as indicated on the Figure. ●, Plot of v, the number of μm-moles of 2-naphthyl sulphamate formed in 2 hr., against the steroid concentration; ○, plot of $1/v$ against the steroid concentration.

Table 1. *Effect of bilateral adrenalectomy and ovariectomy on the ability of rat-liver preparations to synthesize 2-naphthyl sulphamate*

Details of the assay are given in the text and the activities are expressed as μm-moles of 2-naphthyl sulphamate formed/hr./g. fresh wt. of liver. The values given are means, together with the standard deviations. Both the control and the operated group contained 12 rats.

	Control group	Operated group
Activity without added oxo steroid	169±47	153±42
Activity in 10 μM-oxo steroid	742±171	699±141

$1/(v-v_0)$ against $1/i$ are linear in both cases. With the rat enzyme the intercept on the $1/(v-v_0)$ axis varies with the substrate concentration but the intercept on the $1/i$ axis is constant. The significance of this latter intercept is discussed below. With the guinea-pig enzyme the intercepts on both the axes varied with changes in the substrate concentration.

Effect of endogenous steroids. It seemed possible that the activity of rat arylamine sulphokinase in the absence of added steroid could be due to the presence of endogenous 17-oxo steroids in the preparation and that in the absence of these the activity might disappear. To test this possibility rats were bilaterally adrenalectomized and ovariectomized: after 7 days, during which they were maintained with 1% NaCl in their drinking water, they were killed and the complete enzyme system was prepared as usual from samples of their livers. Such treatment must have considerably lowered the level of the endogenous 17-oxo steroids in the

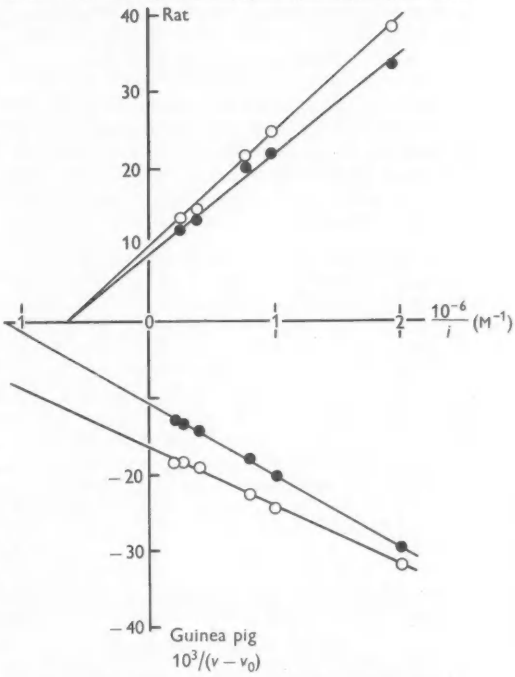

Fig. 6. Reciprocal plot of an experiment corresponding to that shown in Fig. 5. The graph shows the plot of $1/(v-v_0)$, where v_0 and v are the velocities in the absence of and the presence of steroid respectively, against $1/i$, where i is the steroid concentration (M). Concentrations of 2-naphthylamine: 2 mM, ●; 1 mM, ○.

livers of these animals, but there was no significant difference between the enzyme activity of these preparations and those from a similar group of untreated rats. This is shown by the results in Table 1. It would seem therefore that the activity of rat arylamine sulphokinase cannot be due to the presence of endogenous 17-oxo steroids in the enzyme preparations.

Further evidence for this view is given in Fig. 7, which shows that with enzyme preparations from individual animals the activation caused by the addition of 10 μM-3β-methoxyandrost-5-en-17-one is independent of the initial activity of the preparations in the absence of added steroid. If the variations in the initial activities were due to variations in the amounts of endogenous 17-oxo steroids in the preparations then it would have been expected that those preparations with a low initial activity would undergo a greater activation than those with a high initial activity. This is not the case, and again it must be concluded that the activity in the absence of added 17-oxo steroid is not due to endogenous compounds of this type.

DISCUSSION

The method adopted to express the velocity of sulphamate formation (the number of μm-moles of sulphamate formed in the 2 hr. period) may be criticized because the reaction is not of zero order throughout this period. However, the departure from zero order is small and the method appears justifiable. In the experiment illustrated in Fig. 1, for instance, the rates of the control and activated reactions are 35 and 107 respectively when measured as described above; when calculated from the slopes of the linear portions of the progress curves the values are 40 and 112 respectively. The differences between these values, particularly for the activated reaction, are too small to justify the much more complex experimental technique which would have been required to determine in each case the slope of the linear portion of the appropriate progress curve.

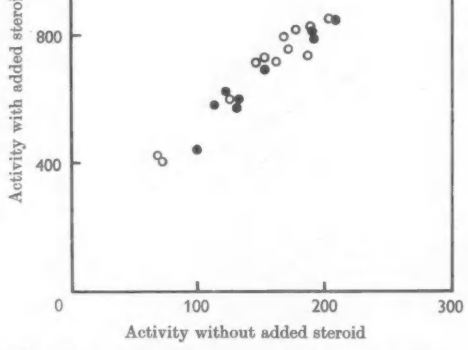

Fig. 7. Comparison of the rates of sulphamate synthesis by enzyme preparations from individual rats in the presence of 10 μM-3β-methoxyandrost-5-en-17-one and in the absence of added steroid. Each point corresponds to determinations with a single enzyme preparation from untreated female rats (●) and from bilaterally ovariectomized and adrenalectomized rats (○).

The atypical shape of the substrate curve for guinea-pig arylamine sulphokinase is interesting. The phenomenon of a critical substrate concentration is of rare occurrence, and apart from its original detection in the hydrolysis of starch by barley amylase (Eadie, 1926) the only other example which appears to have been noted was found in the hydrolysis of phenyl β-glucuronide by mouse-liver β-glucuronidase (Kerr, Graham & Levvy, 1948). With both of these there was apparently a true critical substrate concentration below which no activity whatever could be detected, so that the situation differs from that in guinea-pig arylamine sulphokinase where the substrate curve is sigmoidal at low substrate concentrations. The only immediately obvious explanation of this latter type of effect is that there must be more than one active centre in the enzyme molecule and that combination of the substrate with the first of these centres facilitates the subsequent combination of substrate with the other centres. Whatever be the explanation of the effect it is obvious from the shape of the substrate curve (Fig. 3) that the normal reciprocal plot of the results will be non-linear. However, at concentrations of substrate which are high relative to the apparent critical concentration the experimental results apparently fit an equation of the type

$$v = \frac{V(s-x)}{K_m + (s-x)}, \qquad (1)$$

where x is the apparent critical substrate concentration, as the plot of $1/v$ against $1/(s-x)$ is linear at such substrate levels. This is shown in Fig. 4. It follows from equation (1) that in such a reciprocal plot the intercept on the abscissa will be equal to $-1/K_m$.

The reaction sequence which was previously postulated (Roy, 1960) as an explanation of the activation of 2-naphthyl sulphamate synthesis by 17-oxo steroids was:

However, the experimental findings reported above are not in accord with such a scheme. The important discrepancies are as follows. If the formation of the enol sulphate were rate-limiting then the velocity of sulphamate production via this compound, i.e. $(v-v_0)$, should be independent of the 2-naphthylamine concentration. The plots of $1/(v-v_0)$ against $1/i$ should therefore be independent of changes in the naphthylamine concentration: as shown in Fig. 6 this is not the case. Similarly, if the transfer of sulphate from the enol sulphate to the arylamine were rate-limiting then $(v-v_0)$ would be independent of the steroid concentration, provided that this were sufficiently high to ensure that the rate of enol sulphate production were not limiting. This again is not the case. It is obvious therefore that the reaction mechanism described above is not in accord with the experimental findings and some other explanation of the activation of 2-naphthyl sulphamate synthesis by 17-oxo steroids must be sought. The inhibition of the closely related guinea-pig arylamine sulphokinase should clearly be taken into account in any interpretation of the results, as although the effects in the two species could be unrelated this seems unlikely. A satisfactory explanation of the results must therefore account for both the inhibitory and activatory effect of 17-oxo steroids.

As shown by the results in Figs. 4 and 5, the inhibition of guinea-pig arylamine sulphokinase by 3β-methoxyandrost-5-en-17-one was of the partially competitive type. The reactions which are assumed by Dixon & Webb (1958) to occur in this type of inhibition are:

$$\begin{aligned} E+S &\rightleftharpoons ES \quad \ldots\ldots K_s \\ E+I &\rightleftharpoons EI \quad \ldots\ldots K_i \\ EI+S &\rightleftharpoons EIS \ldots\ldots K'_s \\ ES+I &\rightleftharpoons EIS \ldots\ldots K'_i \\ ES &\xrightarrow{k} E+\text{products} \\ EIS &\xrightarrow{k} EI+\text{products} \end{aligned}$$

These symbols have the following significance: E, arylamine sulphokinase–adenosine 3′-phosphate 5′-sulphatophosphate complex (it is necessary to assume that all the enzyme is present in this form in order to simplify the treatment of the kinetics, and this assumption seems justifiable once the reaction has reached the steady state); S, 2-naphthylamine; I, 3β-methoxyandrost-5-en-17-one; ES, EI and EIS are the appropriate complexes with the dissociation constants K_s, K_i, K'_s and K'_i as indicated. The velocity constant, k, for the breakdown of the binary enzyme–substrate complex is identical with that for the breakdown of the ternary enzyme–steroid–substrate complex. In the following discussion the concentrations of the various reactants are indicated by the appropriate lower-case letters.

On the basis of these assumptions Dixon & Webb (1958) derived for a reaction of this type a velocity equation of the usual form, which can be rearranged to give

$$v = \frac{Vs}{K_s\left\{\frac{K_iK'_s+K'_si}{K_iK'_s+K_si}\right\}+s}. \qquad (2)$$

With guinea-pig arylamine sulphokinase the terms in s would be replaced by corresponding terms in $(s-x)$, where x is the apparent critical substrate concentration. This substitution makes no difference to the general form of the equation.

Equation (2) can only be the velocity equation of an inhibited reaction when K'_s is greater than K_s; that is, when the addition of I decreases the apparent affinity of the enzyme for its substrate. If, on the other hand, the addition of I increases the apparent affinity of the enzyme for the substrate so that K'_s is less than K_s then equation (2) would in fact be the velocity equation of an activated reaction. This single expression can therefore represent either an inhibited or an activated reaction, depending upon the relative values of the dissociation constants K_s and K'_s. This situation might be that found in the arylamine sulphokinases, as the apparent affinities of the enzymes for 2-naphthylamine vary in the expected way (Fig. 3).

It follows from equation (2) that the plots of $1/v$ against $1/s$, or $1/(s-x)$, should be linear at constant values of i. The intercept on the $1/v$ axis will be $1/V$ and will be independent of i; the intercept on the $1/s$ axis will be $-1/KK_s$, where K is the term in K_s, K'_s, K_i and i which appears in the velocity equation, and will therefore vary with i. As shown in Fig. 4, this is in fact the situation found in the inhibition of guinea-pig arylamine sulphokinase and in the activation of rat arylamine sulphokinase.

The velocity in the absence of any added steroid, v_0, is given by the expression

$$v_0 = \frac{Vs}{K_s+s}, \qquad (3)$$

where the symbols have the same significance as those above and where the terms in s are replaced by $(s-x)$ for the guinea-pig enzyme. From these expressions for v and v_0 the following can be derived:

$$\frac{1}{v-v_0} = \frac{K_iK'_s(K_s+s)^2}{VsK_s(K_s-K'_s)}\frac{1}{i}+\frac{(K_s+s)(K'_s+s)}{Vs(K_s-K'_s)}. \qquad (4)$$

From this it follows that the plots of $1/(v-v_0)$ against $1/i$ at constant values of s will be linear.

The second term on the right is the intercept on the $1/(v-v_0)$ axis and will obviously vary with s, as is in fact the case with both the guinea-pig and rat enzymes (Fig. 6). The intercept on the $1/i$ axis is

$$-\frac{(K_s'+s)\,K_s}{(K_s+s)\,K_s'}\,\frac{1}{K_i}.$$

This will also vary with s except when K_s and K_s' are large compared with s, when the expression will reduce to $-1/K_i$. As is shown in Fig. 6, this simplification is apparently valid with rat arylamine sulphokinase as the intercept on the $1/i$ axis is independent of s and gives a value for K_i of 1·5 μM-3β-methoxyandrost-5-en-17-one.

The experimental results are therefore in accord with an explanation of the steroid effects based on the assumed formation of a ternary enzyme–steroid–substrate complex with a dissociation constant of K_s'. Rat arylamine sulphokinase is activated by such a mechanism because K_s' is less than K_s, the dissociation constant of the simple enzyme–substrate complex. Guinea-pig arylamine sulphokinase is inhibited by a similar mechanism because in this case K_s' is greater than K_s. It must be stressed, however, that although the kinetics of the reactions are in accord with this mechanism they cannot prove its occurrence, although they do make the explanation a probable one. If this is indeed the explanation of the mechanism of the action of 17-oxo steroids on arylamine sulphokinases then it is the first example of such a situation, which was discussed on theoretical grounds by Dixon & Webb (1958).

If, as suggested by the data of Fig. 3, 2-naphthylamine does not combine directly with the rat-liver enzyme, the first and fourth reactions in the above-mentioned sequence disappear, with the effect of making infinite the value of K_s in the velocity equations. The resulting expressions for the relationships between $1/v$ and $1/s$ and $1/(v-v_0)$ and $1/i$ are of the same general form and will, in general, give linear plots indistinguishable from those described above, except that in the plot of $1/v$ against $1/s$ the intercept on the ordinate will vary with changes in steroid concentration. Considerably more information will be required to allow the distinction to be made between this situation and the purely competitive one described above.

Whether or not the above explanation is correct, it is obvious that the mechanism previously suggested (Roy, 1960) is incorrect and it is not necessary to postulate the formation of a steroid 17-enol sulphate, or at least not its occurrence in the free state. It is conceivable that such a compound could be implicated in the reaction mechanism now suggested provided that it remained bound to the enzyme. One aspect of the work requires a much more detailed investigation: this is the nature of the linkage between the steroid and the enzyme. Of the steroids so far tested only 17-oxo steroids influenced arylsulphamate synthesis (Roy, 1960). This implies that the D ring of the steroid is involved in the formation of the enzyme–steroid complex, but whether the reaction occurs through the 17-oxo group itself or through the activated 16-methylene group cannot be decided with the information at present available. The observation that 16-hydroxy-17-oxo steroids do not influence sulphamate synthesis (Roy, 1960) perhaps favours the latter view, although steric effects due to the hydroxyl group cannot be excluded. Finally, it should be pointed out that simple cyclic ketones such as *cyclo*pentanone and *cyclo*hexanone do not influence sulphamate synthesis, so that the combination between the steroid and the enzyme cannot simply be a function of the D ring.

The most interesting aspect of the results, whatever their interpretation, is the finding that a 17-oxo steroid can act as a powerful inhibitor of an enzyme in one species and as a powerful activator of the homologous enzyme in another, not very distantly related, species. This is made the more interesting by the fact that the steroid in question is closely related to physiologically active compounds and that similar effects are undoubtedly caused by the active steroids themselves (Roy, 1960). It would seem therefore that these observations are the first to detect species differences in the response to physiologically active steroids at the level of an isolated enzyme. There is as yet no evidence to suggest that the described effects are occurring *in vivo*, but as the concentrations of steroid involved are close to the physiological it would seem that there is no reason why they should not be, provided that the steroid can gain access to the enzymes in the body. Unfortunately the situation *in vivo* cannot be directly investigated as the function of arylamine sulphokinase in the normal animal is unknown.

SUMMARY

1. A method is described for the study of the formation of 2-naphthyl sulphamate by arylamine sulphokinase.

2. The kinetics of the reaction are described, and in particular the kinetics in the presence of 3β-methoxyandrost-5-en-17-one.

3. This oxo steroid strongly inhibits guinea-pig arylamine sulphokinase and activates rat arylamine sulphokinase. Both effects are pronounced at steroid concentrations of 1 μM.

4. Rat-liver arylamine sulphokinase has a very high K_m for 2-naphthylamine, certainly much

greater than for 2 mM-2-naphthylamine. Its apparent affinity for 3β-methoxyandrost-5-en-17-one is high, K_i being 1·5 μM.

5. The corresponding values for guinea-pig-liver arylamine sulphokinase are: K_m, 0·5 mM-2-naphthylamine; K_i, less than 0·2 μM-oxo steroid.

6. An explanation of these effects is suggested. It is based on a mechanism formally similar to the partial competitive inhibition of Dixon & Webb (1958), in which a ternary enzyme–steroid–substrate complex (dissociation constant K'_s) can be formed. The rate of breakdown of this complex to give the reaction products is identical with that of the breakdown of the normal enzyme–substrate complex (dissociation constant K_s). If K'_s is greater than K_s in such a system then the steroid will be an inhibitor of the enzyme; if K'_s is less than K_s the steroid will be an activator.

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Further Experiments on the Inhibition of Aconitase by Enzymically Prepared Fluorocitric Acid

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Peters, Wakelin, Rivett & Thomas (1953) found that fluorocitric acid made by enzymic action inhibits the aconitase in kidney particles more powerfully than does synthetic fluorocitric acid. This finding was in line with the fact that the synthetic fluorocitric acid should be a mixture of four isomers, of which only one or two might be expected to be active. When Morrison & Peters (1954) compared the effects of enzymically and chemically prepared fluorocitrate on a highly purified soluble aconitase, the behaviour of the two was very different. Synthetic fluorocitric acid was a very strong inhibitor, whereas the enzymic fluorocitric acid was much less inhibitory. The inhibition obtained was reversible even after preincubation in the absence of substrate.

These differences were difficult to explain and led to the suggestions either that the synthetic fluorocitric acid did not penetrate properly into the particle, or alternatively that there was some difference in mitochondrial aconitase. The use of another preparation of soluble aconitase has now made it possible to get an inhibition with fluorocitrate which is of the same order as that found in the particle preparation. In addition, a new method of testing the activity of specimens of fluorocitric acid has been devised. Some of these results have already been published in a preliminary form (Peters, 1959).

There are several problems involved: (1) that of the discrepancy between the effect of enzymically prepared fluorocitrate on mitochondrial and soluble aconitase; (2) that of the best method of testing the relative activity of specimens of fluorocitric acid with soluble aconitase; (3) that of the discrepancy between the activity of enzymically prepared and synthetic fluorocitric acid on soluble aconitase; (4) finally, whether the inhibition is truly reversible with enzymically prepared or synthetic fluorocitric acid.

Of these problems only (1), (2) and (4) are considered here. Problem (3) forms the subject of a separate paper (Ward & Peters, 1961).

METHODS AND PREPARATIONS

Chemicals. These were AnalaR where possible; DL-*iso*citric acid (as Na salt at pH 7·4) was prepared from the lactone essentially by the method of Krebs & Eggleston (1944) or Ochoa (1948). Glass-distilled water was used throughout. Phosphate buffer was made by neutralizing KH_2PO_4 solutions with NaOH.

Fluorocitric acid. This was neutralized to pH 7·4, usually

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with NaOH. Where possible the preparations used were made biosynthetically. In some cases, where it was a matter of comparison, to save such preparations, synthetic specimens were used; that these were generally only half as active did not matter in such experiments. During the progress of this work, it was discovered that the maximum activity of specimens of synthetic fluorocitric acid was obtained after an activation by alkali, which is described by Ward & Peters (1958, 1961). The final and crucial experiments were made with enzymically prepared fluorocitrate purified by Dr P. F. V. Ward on silicic acid columns and treated with alkali.

Aconitase. The aconitase preparations were made by the method of Morrison (1954). They were either taken to the final stage of purification (M) or used after the first ethanol precipitation (E). When required they were activated by Morrison's (1954) method based on Dickman & Cloutiers' (1950) discovery; in this an appropriate amount of enzyme (0·2 ml.) was treated with ferrous ammonium sulphate (1·0 mM, 0·3 ml.), to this was added L-cysteine hydrochloride (63·6 mM, 0·75 ml.) neutralized, to pH 7·4, immediately before addition, and the mixture then made up to 2·0 ml. with water. After standing at 0° for 1 hr. amounts of 0·1–0·25 ml. of enzyme solution, depending on the activity, were taken for each cuvette in the experiment. When used without activation 0·3 ml. of enzyme solution was made up to 2·0 ml. with water and 0·25 ml. of this dilution put into each cuvette. The experiments were made by observing the increase of absorption at 240 mμ, which represents the development of the double bond, and so of the formation of *cis*-aconitate from *iso*citrate. The cuvettes were filled in the following order: water (2·0 ml.) or the very dilute *iso*citrate if used for stabilization; buffer solution (0·3 ml.), which was initially tris (2-amino-2-hydroxymethylpropane-1:3-diol), pH 7·7, or phosphate of the same pH; latterly tris (pH 7·4) was used exclusively; fluorocitric acid (neutralized), enzyme solution 0·1–0·25 ml.; and finally water to a total volume of 2·75 ml. By adding the enzyme last, it was exposed to the required concentration of fluorocitrate. The whole was mixed by passing air through the solution. After incubation, sodium *iso*citrate was added as mentioned below.

In addition to the water blank, when the usual carrier is used, three experimental cuvettes can be studied at a time, e.g. the enzyme alone, and the enzyme with two concentrations of fluorocitrate. The solutions were kept in the constant-temperature room in the spectrophotometer usually for 10 min., during which time an equilibrium temperature of 21·9±0·2° was reached. It was better not to use a water-jacketed cuvette holder, as with this only two cuvettes could be used at one time. It was thus more difficult to allow for the gradual loss of activity of the enzyme. After an initial reading, the experiment was best started by adding 0·01 ml. of DL-*iso*citric acid (containing 1·38 μmoles of D-isomer) with an Agla syringe. If this amount is put carefully on the top of the solution in the cuvette, not much interaction takes place until stirring, which was done by passing air. By this method duplicates agreed to within ±0·01. The extinction at 240 mμ was read at 0·5, 1, 2, 3 and 4 min. As described by Morrison & Peters (1954) an initial disturbance, which was non-enzymic, occurred on adding the reagent and largely finished in 0·5 min. The effects were compared between 0·5 and 4 min.

RESULTS

Some observations on the stability of different preparations of aconitase

Purified aconitase. A specimen of Morrison's (1954) purified aconitase (M) at the final stage, which had been kept at −16°, remained active for over four years, though towards the end of this time there was some loss of activity. The specimen had been repeatedly thawed and refrozen. There was little activity in this specimen without activation with Fe^{2+} ions and cysteine, in agreement with Morrison's (1954) observations.

In our hands preparations taken further than the 'first ethanol' precipitate to various intermediate stages towards a final purification lost their activity on freezing and thawing. We have made no attempt to find the reason for this; but we suggest that it is connected with the volume in which the final stage tested is dissolved. Two further preparations taken to the first ethanol stage behaved similarly to the one on which most work was done.

Aconitase at the first ethanol-precipitation stage (E). This was used in the majority of the experiments. As Morrison (1954) showed, purification was accompanied by loss of activity due to removal of Fe^{2+} ions. Hence the purest preparation (M) was almost inactive without treatment with Fe^{2+} ions and cysteine. At stage E, about 50 % of the enzyme is still active without treatment with Fe^{2+} ions and can therefore be used with a simple dilution. When dissolved in water, it was clear and stable for 5 months at −19°, and could be thawed and refrozen with only slight loss of activity. After 6 months' storage, the decrease in activity was under 20 %, but larger losses occurred upon longer storage. It was best to store the preparations in 10 ml. tubes at −19°, which avoids thawing the whole bulk.

The kinetics of the enzyme reaction with aconitase preparation E are considered below. In all the experiments (unless specially mentioned) only observations during the first 0·5–4 min. are reported. After 4·0 min. the observations became progressively vitiated by *cis*-aconitate accumulation, which alters the rate of reaction (see below). The time given was a convenient compromise.

Inhibition of soluble aconitase preparations

Morrison's purified preparation (M). Tests with fluorocitrate specimens in which the activated enzyme was used gave essentially the same results as those of Morrison & Peters (1954); high concentrations of enzymic fluorocitric acid were required to give a substantial inhibition; but the inhibition was somewhat variable; thus 5 and 15 μg. in one case reduced activity by 10 % and in another case

30 μg. was needed for a decrease of 60%; it is a common experience with this type of preparation to find that an increase of fluorocitrate concentration beyond a certain value gives little or no change in the inhibition.

When tris buffer was substituted for phosphate, though the activity of the enzyme alone was reduced (about 22% during the 4 min. period), the inhibition for 5 μg. of fluorocitric acid was increased from 15 to 52% (Figs. 1*a* and 1*b*).

Morrison (1954) found that with M preparations activity was lost in phosphate-buffered solution unless some *iso*citrate was present during the preincubation period. With tris buffer this loss did not occur, and in one experiment, when no *iso*citrate was added during incubation, there was a slight increase in inhibition from 22 to 37%. But the effect of changing to tris buffer seemed to be much less than with the enzyme E.

First ethanol stage (*E*). This contained much of the enzyme in a form not requiring activation by Fe^{2+}–cysteine. Fig. 1*c* shows that when activated with Fe^{2+} ions and cysteine, the effects of fluorocitric acid were much greater than in M, i.e. 71% for 2 μg. of fluorocitrate. As, differing from M, there was no noticeable loss of activity upon incubation for 10 min. without *iso*citrate, this was omitted, and the enzyme was studied also in tris buffer without activation. The effect of as little as 0·002 μg. of biosynthetic fluorocitric acid could be detected under the conditions of test; 0·02 μg. gave an inhibition of 64% similar to that of 10 μg. or more with the M enzyme, i.e. the activity was at least 500 times greater than previously (Fig. 2). Accepting the values as they stand, 50% of the maximum inhibition found experimentally was given by $6{\cdot}5 \times 10^{-9}$ M (6·5 $\mu\mu$M; 0·0035 μg./cuvette), a value which is to be compared with the 58% inhibition induced previously with the M enzyme by 0·16 mM

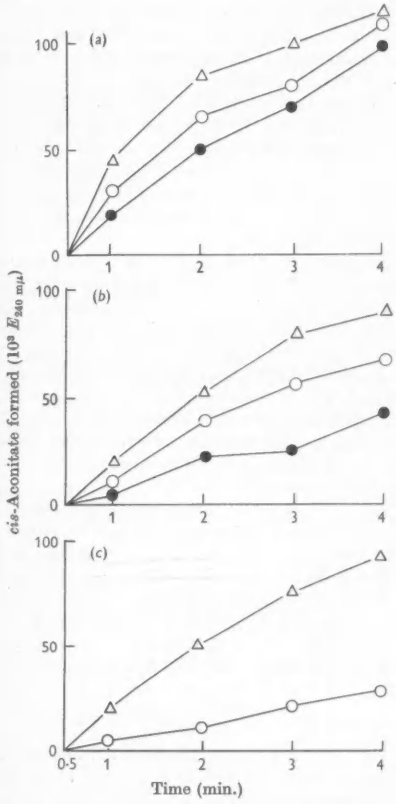

Fig. 1. Comparison of the inhibition induced by 'enzymic' fluorocitric acid on Morrison's purified aconitase (M) and aconitase at the first ethanol stage (E) in phosphate or tris buffer, pH 7·7. Both preparations were activated with Fe^{2+} ions and cysteine as in Morrison's method. The enzyme was preincubated in the presence or absence of fluorocitrate for 10 min. in the presence of dilute DL-*iso*citrate (0·19 mM). The experiment was started by adding DL-*iso*citrate (2·76 μmoles) and observing for 4 min. Comparisons of absorption at 240 mμ due to formation of *cis*-aconitate from 0·5 to 4·0 min. are: (*a*) M in phosphate buffer; (*b*) M in tris buffer; (*c*) E in tris buffer. △, No fluorocitrate; ○, 2 μg. of fluorocitrate; ●, 5 μg. of fluorocitrate.

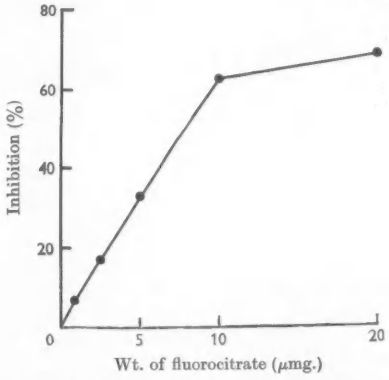

Fig. 2. Inhibition of aconitase at the first ethanol stage in tris buffer (pH 7·4) by enzymic fluorocitric acid (activated). The enzyme was preincubated in the presence or absence of fluorocitrate for 10 min. without *iso*citrate. The experiments were started by adding DL-*iso*citrate (2·76 μmoles), and observing for 4 min. The ordinate scale represents the change in absorption at 240 mμ from 0·5 to 4·0 min., as percentage inhibition.

(Morrison & Peters, 1954). Instead of being less, the inhibition here may be even greater than that on the aconitase in the mitochondrial particles, though the concentration inside such particles *in vitro* is not accurately known. Hence it is clear that a soluble form of aconitase can behave like 'particulate' aconitase, and it is no longer necessary to invoke differences in permeability or in orientation to explain the different concentrations formerly found necessary for particulate and soluble forms of the enzyme.

In a search for the factors which decrease the inhibitory activity of fluorocitrate on the soluble aconitase E, we have investigated the effects of: (1) substituting phosphate for tris buffer which gave a reduction in inhibition to 15% from 47% (Figs. 3*a* and *b*); (2) activation of the aconitase in the usual manner as compared with the non-activated enzyme. This reduced the inhibition. For instance, the percentage inhibition for 0·04 μg. of biosynthetic fluorocitrate (69 μmM) was 57·6% for non-activated and 25% for the preparation treated with Fe^{2+} ions and cysteine; (3) the addition of Fe^{2+} ions and cysteine alone or separately to the cuvettes. The effect of these was variable. In our first preparation they had no appreciable effect, e.g. in the presence of Fe^{2+} alone 0·1 μg. of fluorocitrate inhibited 55%, 0·05 μg. of fluorocitrate inhibited 34%; with cysteine alone 0·1 μg. of fluorocitrate inhibited 43% and 0·2 μg. of fluorocitrate, 62%. With cysteine + Fe^{2+}, 0·1 μg. of fluorocitrate inhibited 39% and 0·2 μg. of fluorocitrate inhibited 55%. In a later preparation, Fe^{2+} and cysteine much reduced the effects of fluorocitric acid. This point evidently requires more work; but it can be stated that the addition of the small amounts of Fe^{2+} ions and cysteine, which might be carried over from an activation, had no effect; (4) the addition of small amounts of *iso*citrate (0·19 mM) during the incubation period. This reduced the inhibition with 0·1 μg. of fluorocitrate from more than 60 to 6%.

The general effect of (1), (2) and (4) is to decrease the inhibition induced by a given amount of fluorocitric acid, though none of them can be responsible for the very large difference in inhibition between M and E. It may be concluded that, practically, for the greatest sensitivity, tests should be done with a preparation at the first ethanol stage of purification, diluted with water and not activated with Fe^{2+} ions, in the presence of tris buffer after a 10 min. incubation with the fluorocitrate in the absence of *iso*citrate.

Effect of temperature. The variation of inhibition with temperature has been studied to make certain that differences of $\pm 0·2°$ do not influence the results. With a jacketed cuvette, the change for $\pm 3·0°$ to $\pm 4·0°$ was not great, from -69% at 21·9° to -56% at 27° and -52% (at 17°) respectively or about 4% per degree (Table 1). In another experiment at 27° the change was to -48%.

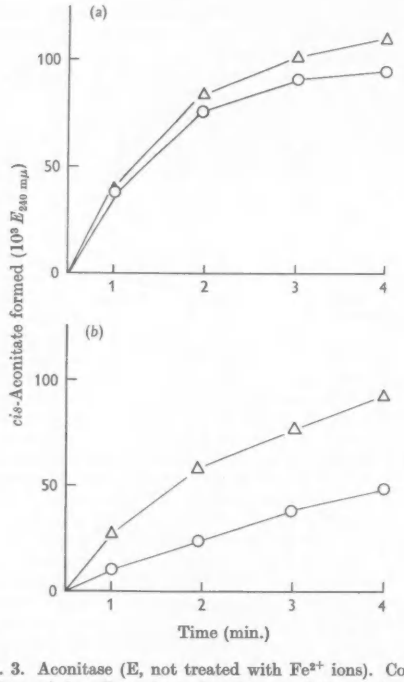

Fig. 3. Aconitase (E, not treated with Fe^{2+} ions). Comparison of the effect of (*a*) phosphate, and (*b*) tris buffer (pH 7·4) on the inhibition by fluorocitrate. The substrate was DL-*iso*citrate. △, Control; ○, 0·2 μg. of synthetic fluorocitrate.

Table 1. *Effect of temperature on the inhibition of aconitase for period 0·5–4 min. after addition of* DL-iso*citrate*

The enzyme control was read against the water blank, then against the enzyme containing 0·01 μg. of fluorocitrate and finally again against water to ensure that there had been no change in activity. For other experimental details, see text.

Temp.	Conditions	Diff. in absorption (10^3 $E_{240\ m\mu}$)	Inhibition* (%)
27°	Control	70, 68	56·5
	Fluorocitrate	30	
17°	Control	59, 53	52
	Fluorocitrate	27,	

* 100 − % difference.

Effect of pH. A fall of 10% in the activity of the enzyme was observed on changing from pH 7·7 to 8·3. With tris buffer at pH 7·95 instead of pH 7·4 a change in the inhibition was found, from 69 to 66%. With tris buffer after bringing to pH 6·8, there was an increase of 4% in enzymic activity, i.e. within experimental error. The inhibition was changed from 67 to 63%. It was considered that the effect of smaller variations in pH could be neglected.

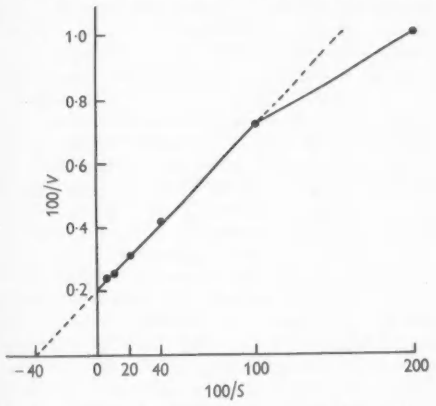

Fig. 4. Apparent K_m for aconitase E. Reaction *iso*citrate → *cis*-aconitate in tris buffer, pH 7·4.

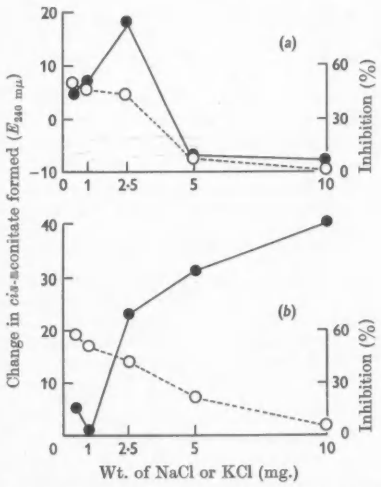

Fig. 5. Effect of KCl and NaCl on the inhibition of aconitase E produced by enzymic fluorocitrate (0·01 μg.) and on the enzymic activity. Each point represents the difference in the activity or the amount of *cis*-aconitate formed from DL-*iso*citrate (2·78 μmoles) for the period 0·5–4·0 min. in the presence and absence of the salts indicated. Preparations were preincubated for 10 min. (*a*) KCl, 10 mg. (48·5 mM); (*b*) NaCl, 10 mg. (62·4 mM). —, Effect on aconitase activity; - - -, change in percentage inhibition.

Kinetic behaviour. Fig. 4 gives the apparent K_m for the aconitase E for the reaction *iso*citrate → *cis*-aconitate in tris buffer (pH 7·4) plotted in the conventional way as $100/V$ against $100/S$. Reckoned as DL-*iso*citrate, K_m is 4·73 mM, making 2·36 mM for the D-*iso*citrate component. This is to be compared with Morrison's value of 0·48 mM, which was done in the presence of phosphate at pH 7·7. It is to be noted that the value for the lowest concentration of the substrate falls off the line. This has not been explained.

Effects of variations in the salt content. Though this has not been investigated extensively, some facts about the effects of salt concentration on the degree of inhibition by fluorocitrate were obtained, because it is sometimes necessary to assay tissue extracts with variable salt contents. In general (Fig. 5) an increase of both NaCl and KCl lowered the inhibition observed with a small amount of fluorocitrate; the presence of 0·0624 M-NaCl and 0·0485 M-KCl reduced this almost completely, and about 50 times the amount of fluorocitrate was then needed for the same degree of inhibition.

There were interesting differences between NaCl and KCl in their effects on aconitase alone; increasing amounts of NaCl increased the activity, whereas KCl gave a more complicated picture, first increasing and then decreasing activity. It is clear that the salt content must be controlled when testing for fluorocitrate concentration with aconitase, and that more work is needed to understand what is happening.

Reversal of the inhibition by fluorocitrate and the effect of pre-formed cis*-aconitate and citrate on the reversal.*

Many experiments were done similar to those of Morrison & Peters (1954) in which more *iso*citrate was added after 4–5 min. to ascertain the amount by which the inhibition was reduced on increasing the concentration of substrate. This is important because the possibility of finding an antidote to fluoroacetate poisoning turns to some extent on whether the inhibition by fluorocitrate is reversible. The experiments made in this series are not given in detail here; in all experiments there was indication of reversal by a closer approximation of the curves of reaction, and sometimes the reversal was complete. But the results were variable, and this led to a closer examination of the phenomena.

In our original experiments, the effect on the subsequent behaviour of any *cis*-aconitate or citrate

formed during the initial period of exposure to the lower *iso*citrate concentration was neglected. If, however, small amounts of *cis*-aconitate (or citrate) have an appreciable inhibitory effect on the subsequent course of the reaction *iso*citrate → *cis*-aconitate when more *iso*citrate is added, then the reversal of the inhibition might be only apparent. The greater amount of *cis*-aconitate formed in the control might slow the reaction enough to make the rate identical with that of the cuvette containing fluorocitrate. This would mean that the inhibition was less competitive than we had thought. It could also account for some of our variable results. The matter has been tested by starting the reaction with some *cis*-aconitate or citrate present.

An addition of 38·7 μmoles of citrate had little effect on the rate of reaction of *iso*citrate; but it was different with *cis*-aconitate. Fig. 6 shows that 15 μg. (0·086 μmole) and 25 μg. (0·14 μmole) of *cis*-aconitic acid (neutralized with NaOH) induce large effects on the rate of reaction of 2·6 μmoles of DL-*iso*citrate with aconitase. It can be calculated that at the end of the 4 min. incubation there may sometimes be a difference between the sample inhibited by fluorocitrate and the control sample of approx. 0·052 μmole (10 μg.) of *cis*-aconitic acid. The extra *cis*-aconitic acid in the control sample could slow the rate of the subsequent reactions occurring after the further addition of *iso*citrate, so that the control rate became identical with that of the inhibited reaction; the reversibility described above would be then only an apparent reversibility. The degree of inhibition for 0·005 μg. of fluorocitrate was 50 or 54 %, i.e. unchanged within the limits of error by the effect of *cis*-aconitate.

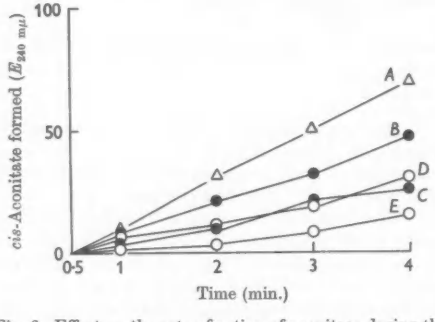

Fig. 6. Effect on the rate of action of aconitase during the first 4 min. of adding initially small amounts of DL-*cis*-aconitate. Experimental details were as for Fig. 2. All contained 2·76 μmoles of DL-*iso*citrate. *A*, Control; *B*, 0·086 μmole of *cis*-aconitate; *C*, 0·086 μmole of *cis*-aconitate + 0·005 μg. of fluorocitrate; *D*, 0·144 μmole of *cis*-aconitate; *E*, 0·144 μmole of *cis*-aconitate + 0·005 μg. of fluorocitrate.

In view of these results it became essential to study the inhibition in the presence of increasing concentrations of *iso*citrate added initially. This should show decisively whether increasing concentrations of substrate reversed the effect. An amount of fluorocitrate (0·005 μg.) was used which would produce an inhibition of 50 % of the maximum (see Fig. 6). In Fig. 7 the rates of formation of *cis*-aconitic acid over the 3·5 min. period in the presence and absence of fluorocitrate have been plotted against the initial amounts of DL-*iso*citrate added. This method of plotting was found more satisfactory than any other. With increasing *iso*citrate concentration there was a decreased inhibition; but the reversal was much less marked than in our earlier experiments. It appears therefore that the suspected effect of small amounts of *cis*-aconitate formed during the first few minutes was influencing the previous experiments. In fact in the presence of 208 μmoles of *iso*citrate, added initially, there is still an inhibition of 8 % under these conditions. Hence it is much more difficult to reverse the inhibition than was previously thought. *In vivo* the necessary concentration of citrate for reducing the inhibition to 8 % could not

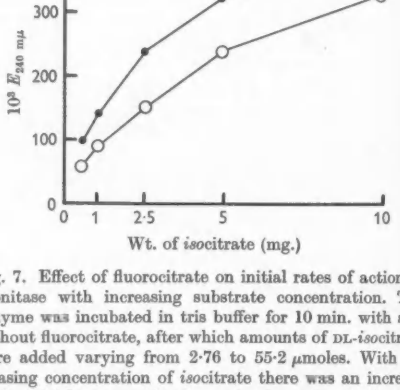

Fig. 7. Effect of fluorocitrate on initial rates of action of aconitase with increasing substrate concentration. The enzyme was incubated in tris buffer for 10 min. with and without fluorocitrate, after which amounts of DL-*iso*citrate were added varying from 2·76 to 55·2 μmoles. With increasing concentration of *iso*citrate there was an increase of the initial non-specific change in absorption in the first 0·5 min.; for instance, whereas with addition of 5·52 μmoles this was 40, with 27·6 μmoles it became 150 and with 110·4 μmoles 250. After disregarding the first 0·5 min. the readings became linear and the two curves show the differences in absorption for amounts of *iso*citrate up to 55·2 μmoles over the remaining 3·5 min. between the controls (●) and samples treated with 0·005 mg. of fluorocitrate (○). It is clear that there is a reversal, but that this is not so marked as was thought previously. Even with 208 μmoles the reversal was not complete. (10 mg. of *iso*citrate ≡ 52·1 μmoles.)

be reached, as this would have to be some nine times that of *iso*citrate.

Fig. 7 indicates that it is difficult to reverse the inhibition *in vitro* under these conditions. However, it must be stressed that the reactions with aconitase are complex. This is shown by the fact that if the amount of enzyme is much increased the amount of *cis*-aconitate formed reaches a lower maximum, and then actually decreases as the reaction proceeds, owing presumably to the formation of citrate, which does not absorb at 240 mμ.

A few experiments were made on the amount of citrate formed after 4 and 7 min. of reaction. The amounts of citrate present were small, not more than 0·2 μmole/cuvette; such amounts did not increase in this time with increasing concentrations of *iso*citrate. The *cis*-aconitate is evidently not always converted into citrate immediately.

L$_a$(+)-iso*Citrate as substrate.* As all the previous experiments were done with DL-*iso*citrate, some of the main points were checked with a specimen of L$_a$(+)-*iso*citrate for which we are indebted to Dr H. B. Vickery. In every case tried the reaction rate for a given amount of L$_a$(+)-*iso*citrate was approximately double that with the DL-compound, so that the unnatural isomer did not influence these results. There was no difference in the degree of inhibition with fluorocitrate. From this we conclude that the use of the DL-*iso*citrate has not affected our results.

DISCUSSION

The results appear to dispose of some of the difficult discrepancies between the inhibition by fluorocitrate of mitochondrial and of soluble aconitase, as they show that a form of soluble aconitase can be very sensitive to fluorocitrate inhibition. The values reached in these experiments are of the order of those found with the phosphorus-containing insecticides and cholinesterase. On the other hand, an exact evaluation of the conditions inside the mitochondria is difficult. Since with the particle preparations we worked in 1 % KCl so far as the bulk solution was concerned, the inhibitory action of the fluorocitrate would be much diminished if the salt concentration inside the mitochondrion was the same. Within the mitochondrion again the fluorocitrate concentration is unknown; but the enzyme *in vivo* could be more sensitive even than in these tests. A calculation indicated that citrate metabolism in pigeon-brain tissue *in vivo* was blocked by about 0·1 μg. of fluorocitrate/g. of wet tissue. *In vivo* also, accumulation of citrate reduced the inhibition, which could explain the reduction reported by Chenoweth (1949) in the effect of a second dose of fluorocitrate when given 24 hr. later than the first. Amounts of citrate higher than 20·8 μmoles/g. have not been found after poisoning animals with these compounds, and such concentration would not be enough to cause reversal if the behaviour were the same as with aconitase E.

So far as the action of aconitase itself is concerned, these experiments raise more problems than they solve, because they have been oriented solely to the problem of how to test for fluorocitrate. Some of the problems may, however, be mentioned. First, there seems to be a real difference so far as inhibition is concerned between aconitase M and E, which is not just a matter of the amount of Fe^{2+} and cysteine present. If this is so, then the reconstituted enzyme would differ in some way from that in the tissues.

Secondly, it is curious that within the first few minutes of the reaction the amount of citrate formed does not correspond with the increase of *cis*-aconitate when the concentration of *iso*citrate is increased. This is consistent with the idea of the intermediate carbonium ion in aconitase, as set forth most recently by Dickman & Speyer (1954) and Englard & Colowick (1957); but it does not seem consistent with the idea that some citrate can be formed directly without the formation of *cis*-aconitate, unless we take the view that the addition of water to the intermediate complex is more difficult than elimination to the anhydrous *cis*-aconitate.

The changes of activity induced by salts are of theoretical interest and deserve much more study, certainly with change in pH and with other ions. It is possible indeed that there might appear changes in optimum pH similar to those found in different buffers for aconitase (Morrison, 1954) or for fumarase with different inhibitors (Massey, 1953). In order to get a proper picture, however, simultaneous observations will be needed of the amounts of all three tricarboxylic acids, taking into account the large influence of small amounts of *cis*-aconitate.

SUMMARY

1. A new method has been worked out for testing the activity of fluorocitrate on soluble aconitase. In this a cruder enzyme preparation is used, taken to an intermediate stage of fractionation, which does not require reactivation with ferrous salts and cysteine.

2. When this preparation was used in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer solution instead of phosphate solution, 50 % inhibition was observed with fluorocitrate (enzymically synthesized) at a concentration of 6·5 μmM.

3. This large increase in the inhibitory activity observed means that there is no longer a discrepancy between the effects of fluorocitrate upon aconitase in particle preparations and in a soluble form. It makes unnecessary the hypothesis of

specific concentration or orientation previously proposed.

4. The inhibitory effects were much reduced by the presence of phosphate or by preincubation with 5 mM-*iso*citrate.

5. In the presence of sodium chloride the activity of the aconitase increased. With potassium chloride there was an increase followed by a fall. An increase in the concentration of both salts much reduced the inhibition of fluorocitrate.

6. The percentage inhibition with fluorocitrate was the same for L-(+)-*iso*citrate (D), though the rate for a given amount of *iso*citrate was doubled. There was no indication that the inactive isomer was an inhibitor.

7. Variation of temperature within $\pm 4°$ was about 4% per degree. Variations in pH of $\pm 0{\cdot}6$ caused little change in fluorocitrate inhibition, though a fall in activity was found on making more alkaline.

Thanks are due to Miss P. Ivall for efficient technical assistance, and to Miss R. Shawdon for some of the preliminary work; to Dr P. F. V. Ward for the final purification of the fluorocitrate used, to others in the Department for advice and to Dr D. H. Treble for reading the MS. Thanks are also due to Professor F. G. Young, F.R.S., for providing facilities for the finish of this work.

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The Metabolites of Piperic Acid and Some Related Compounds in the Rat

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Human urine contains many compounds which give coloured derivatives with Altman reagent on paper chromatograms (Acheson & Dearnaley, 1960; Smith, Paul, McGeer & McGeer, 1959; Armstrong, Shaw & Wall, 1956). This reagent (4-dimethylaminobenzaldehyde in acetic anhydride) reacts with aroyl-glycines and similar compounds to give coloured oxazolones, a number of which have been synthesized and their absorption spectra measured (Acheson, Booth, Brettle & Harris, 1960). 5-Phenylpenta-2:4-dienoylglycine gives, when heated with the reagent on paper chromatograms, a red–purple colour similar to those given by compounds 54 and 55, which were found in human urine by Acheson & Dearnaley (1960), but the three compounds had different R_F values. It was possible that compounds 54 and 55 were derived from a substituted 5-phenylpenta-2:4-dienoic acid which is a dietary constituent and excreted as the glycine conjugate. In order to test this in connexion with piperic acid, which occurs as derivatives in pepper, piperoylglycine was prepared and the metabolism of piperic acid has been investigated in the rat.

MATERIALS AND METHODS

All melting points are uncorrected, and the absorption spectra were determined in methanol solution unless otherwise specified.

Piperonylic acid, m.p. 226–227°, $\lambda_{max.}$ 293 and 256 mμ (ϵ 6400 and 7500 respectively), was prepared by the oxidation of piperonal with neutral potassium permanganate (*Organic Syntheses*, 1943). 3:4-Methylenedioxyhippuric acid, m.p. 178°, was prepared as described previously by Acheson *et al.* (1960), and 3:4-methylenedioxycinnamic acid, m.p. 238°, was obtained from malonic acid and piperonal (*Organic Reactions*, 1942).

3:4-*Methylenedioxycinnamoylglycine*. The cinnamic acid (1·0 g.) was covered with ethyl aminoacetate (0·8 g.) in dry

tetrahydrofuran (6 ml.) and a solution of di*cyclo*hexylcarbodi-imide (1·5 g.) in tetrahydrofuran (3 ml.) was added (cf. Sheehan & Hess, 1955). The mixture was well shaken and kept for 3½ hr., and the precipitate collected. The filtrate was evaporated to dryness and the residue extracted with ethyl acetate (2 × 10 ml.). The combined extracts were washed with dilute aqueous hydrochloric acid and sodium carbonate and dried. Ethyl 3:4-methylenedioxycinnamoylaminoacetate (0·42 g.), m.p. 135° (cf. Acheson *et al.* 1960), was obtained as colourless crystals when the residue, obtained on evaporating the ethyl acetate, was triturated with light petroleum (b.p. 80–100°) and left overnight. Hydrolysis of the ester (Acheson *et al.* 1960) gave the glycine which separated from dioxan as colourless crystals, m.p. 210°.

Piperoylglycine. Piperic acid, m.p. 213–215°, $\lambda_{max.}$ 226 mμ (ε 18 000), inflexions at 329, 304 and 248 mμ (ε 24 000, 20 000 and 15 000 respectively), was prepared from piperonal according to Lohaus (1929) and was condensed with ethyl aminoacetate as above. The crude ethyl piperoylaminoacetate (21% yield) had m.p. 123–125°, and four recrystallizations from aqueous 50% ethanol gave pale-yellow needles, m.p. 137° (Found: C, 63·9; H, 5·8; N, 4·1. $C_{16}H_{17}O_5N$ requires C, 63·4; H, 5·6; N, 4·6%). This ester was also obtained in 17% yield from piperoyl chloride, m.p. 203–205° [Lohaus (1929) gives m.p. 180°], prepared from the acid and thionyl chloride, and excess of ethyl aminoacetate. Hydrolysis of the ester (0·22 g.) in warm dioxan (4 ml.) was effected by N-sodium hydroxide (1·46 ml.) in 2 hr. at room temperature (cf. Harington & Mead, 1935). The pH was then adjusted to 7 with 2N-hydrochloric acid and the solvents were removed *in vacuo*. The residue was shaken with ethyl acetate (3 × 8 ml.), and the combined solutions were extracted with saturated aqueous sodium bicarbonate (2 × 5 ml.). The bicarbonate solution was extracted with ether and subsequently acidified, when crude *piperoylglycine* (0·06 g.), m.p. 192–197°, was precipitated. Four recrystallizations from aqueous 50% methanol gave yellow plates, m.p. 200–204° (Found: C, 61·1; H, 4·7; N, 5·0. $C_{14}H_{13}O_5N$ requires C, 61·0; H, 4·7; N, 5·1%). It had $\lambda_{max.}$ 336 and 243 mμ (ε 20 000 and 14 000 respectively) and inflexions at 310 and 255 mμ (ε 15 000 and 13 000 respectively).

Treatment of this acid with 4-dimethylaminobenzaldehyde and acetic anhydride (Acheson *et al.* 1960) gave a deep-red crystalline material which after six recrystallizations from benzene reached a constant m.p. 205–206°, and the ultraviolet-absorption spectrum in ethyl acetate was not changed by the last crystallization. It showed $\lambda_{max.}$ 488 and 363 mμ (ε 66 000 and 22 000) and after acidification with concentrated sulphuric acid $\lambda_{max.}$ 426, 345 and 308 mμ (ε 45 000, 15 000 and 13 000 respectively). Although sufficient material for analysis was not obtained, the method of preparation and the absorption spectrum suggest that the material is largely the expected oxazolone.

Animals, diet and dosage. Rats (70–100 g.) were kept in pairs in metabolism cages with an unrestricted water supply. They were placed in feeding cages twice daily for 30 min. periods during which time they did not urinate. Their normal diet was that supplied by Herbert C. Styles (Bewdley) Ltd. (diet 41). Each rat received piperic acid (50 mg.), 3:4-methylenedioxybenzoic acid (42 mg.), 3:4-methylenedioxycinnamic acid (32 mg.) or 3:4-methylenedioxycinnamoylglycine (32 mg.) mixed with rat-cake (20 g.), which was consumed during the experimental period of 48 hr. Piperoylglycine (10 mg.) in aqueous sodium bicarbonate (0·75 ml.) was fed by stomach tube once daily. The urine was collected for 48 hr.

Extraction of the urine and chromatographic procedures. The urine samples and cage-washings were saturated with ammonium sulphate, acidified to pH 1–2 with hydrochloric acid, and extracted (3 × half the sample volume) with ethyl acetate close to 0° (cf. Armstrong *et al.* 1956). The combined extracts were dried (Na_2SO_4), filtered and evaporated to about 1% of their original volumes, and 25 μl. was chromatographed on Whatman no. 1 paper in solvent *A* (Table 1) in the first dimension and solvent *B* in the second. The chromatograms were examined under a Hanovia ultraviolet lamp emitting 90% of its radiation at 2537 Å, and were subsequently treated with Altman reagent. The identities of the methylenedioxy compounds on the urine chromatograms were shown by comparing their R_F values with those of the authentic materials and also by co-chromatographing the authentic materials with the urine extracts and observing the increase in intensity of the spots. Rough estimates of some of the compounds were made by comparing visually the spots on the urine chromatograms with those produced by known amounts of the authentic materials chromatographed under identical conditions. All chromatograms were run in duplicate and were repeated if good agreement was not obtained. The stability of the methylenedioxy compounds in the urine

Table 1. *Chromatographic behaviour of the authentic methylenedioxy compounds*

Solvents and times of run: *A*, propan-2-ol–aq. ammonia soln. (sp.gr. 0·88)–water (6:3:1, by vol.) (8 hr.); *B*, aqueous 20% potassium chloride (1½ hr.); *C*, butan-1-ol–acetic acid–water (4:1:1) (5–6 hr.); *D*, benzene–propionic acid–water (2:2:1), organic phase (2½ hr.); *E*, propan-2-ol–ammonia–water (8:1:1) (8 hr.). The ascending method on Whatman no. 1 paper was used.

Compound	Colour in ultraviolet light	Colour with Altman reagent	R_F in solvent				
			A	*B*	*C*	*D*	*E*
3:4-Methylenedioxybenzoic acid	Green fluorescence	None	0·81	0·63	0·92	0·96	0·49
3:4-Methylenedioxycinnamic acid	Blue	None	0·87	0·25	0·93	0·98	0·48
Piperic acid	Green–blue	None	0·90	0·05	0·87	0·99	0·46
3:4-Methylenedioxybenzoylglycine	Faint blue	Yellow–orange	0·84	0·73	0·84	0·43	0·43
3:4-Methylenedioxycinnamoylglycine	Bright blue	Orange–red	0·76	0·25	0·80	0·50	0·35
Piperoylglycine	Green–blue	Red–purple	0·87	0·08	0·88	0·56	0·43
54	Absorbs	Red–purple	—	—	0·30	0·00	0·02
55	Absorbs	Red–purple	—	—	0·21	0·00	0·02

Table 2. *Metabolites of methylenedioxy compounds in rat urine*

The compounds indicated were detected on paper chromatograms. + Indicates present, + + present in greater amount and − not detected. Piperic acid was not detected in any of the urines.

	Compound in urine				
Compound fed	3:4-Methylenedioxybenzoic acid	3:4-Methylenedioxycinnamic acid	3:4-Methylenedioxybenzoylglycine	3:4-Methylenedioxycinnamoylglycine	Piperoylglycine
3:4-Methylenedioxybenzoic acid	+	−	+	−	−
3:4-Methylenedioxycinnamic acid	−	−	+ +	+	−
Piperic acid	+	−	+ +	+	−
3:4-Methylenedioxycinnamoylglycine	−	+	+	+	−
Piperoylglycine	−	−	+	+	+

was checked by adding the compounds to a sample of rat urine and working up one half of the urine immediately and the other half after a delay of 72 hr. Chromatograms of equivalent amounts of the extracts were identical. In a similar way the stability of the methylenedioxy compounds in the rat-cake for 72 hr. was ascertained, and the recovery of added 3:4-methylenedioxybenzoic acid and its glycine conjugate from rat urine shown to be about 80%.

RESULTS AND DISCUSSION

The results of the feeding experiments are given in Table 2. None of the methylenedioxy compounds could be detected in normal rat urine. No piperoylglycine could be detected as a metabolite of piperic acid, and the results (Table 2) suggest that four carbon atoms of the side chain can be eliminated in two stages through the corresponding cinnamic acid; a simultaneous direct removal of the four carbon atoms is not excluded. These results are in line with the conversion, by the rabbit, of 5-phenylpenta-2:4-dienoic acid into benzoic acid and cinnamic acid and the corresponding glycine conjugates (Friedman & Mai, 1931). About 40% of the administered piperic acid was eliminated as 3:4-methylenedioxyhippuric acid, and about 0·3% as 3:4-methylenedioxybenzoic acid, within 72 hr. of the beginning of the experiment.

As the side chains of piperic acid and 5-phenylpenta-2:4-dienoic acid are oxidized so readily in the rabbit it is perhaps unlikely that compounds analogous to piperoylglycine will be found in normal human urine. Although the colour developed by piperoylglycine with Altman reagent is similar to that produced by the compounds 54 and 55 present in human urine the chromatographic properties of the three compounds are widely different.

SUMMARY

1. 3:4-Methylenedioxy-benzoic acid and -cinnamic acid, and piperic acid, and their glycine conjugates have been synthesized.

2. None of these compounds was detected in the urine of rats fed with the standard diet and the three acids were partially metabolized to 3:4-methylenedioxyhippuric acid.

3. The chromatographic properties of piperoylglycine are not identical with two other compounds giving a similar Altman reaction and which are present in human urine.

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Uncoupling Reagents and Metabolism

3. EFFECTS OF SALICYLATE AND 2:4-DINITROPHENOL ON THE INCORPORATION OF ^{14}C FROM LABELLED PYRUVATE INTO THE SOLUBLE INTERMEDIATES OF ISOLATED RAT TISSUES*

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Salicylate and 2:4-dinitrophenol produce effects on the incorporation of radioactivity from labelled glucose and acetate into the soluble metabolic intermediates of isolated rat tissues which are explicable in terms of an uncoupling action on oxidative-phosphorylation processes (Smith & Moses, 1960). No qualitative differences between the actions of the two substances were detected. In the present work, [3-^{14}C]pyruvate being used as a labelled substrate, salicylate, but not 2:4-dinitrophenol, was found to increase the incorporation of ^{14}C into glutamic acid in isolated preparations of rat liver and kidney. Glutamic–pyruvic-transaminase activity in rat serum was inhibited *in vitro* by salicylate but was not affected by 2:4-dinitrophenol. A preliminary account of part of this work has been published (Huggins, Moses & Smith, 1960).

EXPERIMENTAL

The methods used for the preparation of the tissues (liver, kidney, brain and heart muscle), for the incubation with the labelled substrates in the presence or the absence of either 5 mM-salicylate or 0·5 mM-2:4-dinitrophenol (DNP) and for the chromatographic separation and measurement of radioactivity in the soluble intermediates were those described by Smith & Moses (1960). Sodium [3-^{14}C]pyruvate (3·62 μc/μmole) and sodium L-[^{14}C]-glutamate (3·83 μc/μmole) were obtained from The Radiochemical Centre, Amersham, Bucks. The labelled glutamate was purified before use by two-dimensional paper chromatography with phenol–water (72%, w/w) as the first solvent and butan-1-ol–propionic acid–water (45·9:23·4:30·6, by vol.) as the second. Approximately 2–3% of the purified material decomposed during the experiments but, with the exception of γ-aminobutyric acid, which formed less than 10% of the decomposition products, the radioactive compounds formed were not metabolized by the kidney preparation used and have not been included in the results.

Glutamic–pyruvic-transaminase and glutamic–oxaloacetic-transaminase activities were measured in the presence or the absence of either 5 mM-salicylate or 0·5 mM-DNP, by the method of Reitman & Frankel (1957), in pooled serum obtained from adult rats of the Wistar strain.

RESULTS

Total incorporation and distribution of ^{14}C from [3-^{14}C]pyruvate in the soluble intermediates. The radioactivity measured was that present in the soluble intermediates. The ^{14}C incorporated into substances such as glycogen or proteins, which were insoluble in aqueous ethanol, or that evolved as CO_2, was not estimated.

The total carbon from the labelled pyruvate which was incorporated into the soluble intermediates of liver, kidney, brain and heart muscle in the presence or the absence of either 5 mM-salicylate or 0·5 mM-DNP is given in Table 1. Brain and kidney were the most active tissues and both uncoupling reagents reduced the incorporation of pyruvate carbon atoms in all the four tissues by about 20–40%. The effect of salicylate was more

Table 1. *Incorporation of ^{14}C from [3-^{14}C]pyruvate into the soluble intermediates of rat tissues in the presence or the absence of either 5 mM-salicylate or 0·5 mM-2:4-dinitrophenol*

Tissues were cut into blocks and incubated aerobically for 3 hr. at 37° with [3-^{14}C]pyruvate of known specific activity. Tissues were killed and extracted with ethanol, the extracts were chromatographed and the radioactivity was measured in the separate metabolites. From the quantity of ^{14}C present in the metabolites the total utilization of pyruvate was calculated, and is expressed below as μmoles of pyruvate incorporated into the soluble fraction of 25 mg. of dry tissue in 3 hr. Values are calculated from the sum of the ^{14}C present in the metabolites, in the cell extract and in the medium.

	Pyruvate incorporation (μmoles)		
Tissue	Control	With salicylate	With DNP
Liver	1·4	1·1	1·0
Kidney	2·3	1·5	1·6
Brain	3·4	2·6	2·0
Heart muscle	1·2	1·0	1·1

* Part 2: Moses & Smith (1960).

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pronounced than that of DNP with kidney and heart muscle, and less with liver and brain.

The results in Table 2 show the amounts of ^{14}C incorporated into the soluble intermediates of the four tissues in the presence or the absence of salicylate and DNP.

In the absence of the inhibitors the qualitative pattern of incorporation from the labelled pyruvate was similar for all the tissues. Radioactivity was found in compounds, such as alanine and lactic acid, which were derived from pyruvate itself by transamination and dehydrogenation reactions.

Table 2. *Metabolism of* [3-^{14}C]*pyruvate by isolated rat tissues in the presence or the absence of salicylate and 2:4-dinitrophenol*

Each flask contained 1 ml. of tissue suspension (20 mg. dry wt. of liver, 11·1 mg. dry wt. of kidney, 11·5 mg. dry wt. of brain or 10·0 mg. dry wt. of heart muscle) and was incubated with 10 μc of the labelled pyruvate for 3 hr. at 37° in the presence or the absence of either 5 mM-salicylate or 0·5 mM-DNP. Radioactivity is expressed as 10^{-3} counts/min./25 mg. dry wt. of tissue and is the sum of the ^{14}C present in the cell extract and the medium.

Tissue ...	Liver			Kidney		
Uncoupling reagent ...	None	Salicylate	DNP	None	Salicylate	DNP
Alanine	323	260	219	300	140	150
γ-Aminobutyric acid	0	0	0	64	4·3	25
Aspartic acid	19	20	25	100	79	52
Glutamic acid	36	69	37	260	320	220
Glutamine	0	0	0	0	0	0
Leucine or isoleucine*	0	0	0	43	28	34
Methionine†	6·4	14	11	24	0	0
Threonine	2·9	3·4	4·6	0	0	0
Glucose‡	34	25	0	67	24	8·1
α-Oxoglutaric acid	0	0	0	0	0	0
Citric acid	21	16	2·1	6·8	27	24
Fumaric acid	4·9	0	6·8	15	14	25
Glyceric acid	0	0	0	85	40	51
Lactic acid	200	92	130	136	57	150
Malic acid	16	27	22	0·4	0	46
Succinic acid	3·4	9	13	13	0	0
Total in						
Amino acids	390	370	300	790	570	480
Organic acids	250	140	170	260	140	300
Glucose	34	25	0	67	24	8·1
Unidentified compounds	5·4	9·7	4·7	26	25	29
Soluble fraction	680	540	470	1100	760	820

Tissue ...	Brain			Heart muscle		
Uncoupling reagent ...	None	Salicylate	DNP	None	Salicylate	DNP
Alanine	140	87	130	120	3·5	4·7
γ-Aminobutyric acid	130	14	47	0	0	0
Aspartic acid	270	200	51	16	92	110
Glutamic acid	730	570	220	130	83	130
Glutamine	3·6	2·3	2·3	0	0	0
Leucine or isoleucine*	44	46	92	99	62	66
Methionine†	16	11	1·3	34	17	14
Threonine	12	0	2·5	0	0	0
Glucose‡	25	0	0	0	0	0
α-Oxoglutaric acid	46	0	0	0	0	0
Citric acid	71	100	97	4·6	0	3·1
Fumaric acid	0	0	0	0	0	0
Glyceric acid	76	88	120	73	38	100
Lactic acid	42	21	37	36	33	10
Malic acid	23	14	27	2·1	1·1	4·2
Succinic acid	20	82	40	13	17	5·1
Total in						
Amino acids	1300	930	550	410	260	320
Organic acids	280	300	320	130	90	120
Glucose	25	0	0	0	0	0
Unidentified compounds	70	86	95	80	72	130
Soluble fraction	1700	1300	960	620	420	570

* Contains trace of phenylalanine.
† Contains trace of valine.
‡ Contains trace of serine or glycine or both.

Table 3. *Percentage distribution of* ^{14}C *from* [3-^{14}C]*pyruvate in the soluble intermediates of isolated rat tissues in the presence or the absence of salicylate or 2:4-dinitrophenol*

Details were as given for Table 2. Results are expressed as % of the total ^{14}C present in the sum of all the soluble intermediates.

Tissue	Liver			Kidney		
Uncoupling reagent ...	None	Salicylate	DNP	None	Salicylate	DNP
Amino acids	57	69	64	72	75	59
Glutamic	5	13	8	24	42	27
Alanine	48	48	47	27	18	18
Aspartic	3	4	5	9	10	6
Organic acids	37	26	36	24	18	37
Lactic acid	29	17	28	12	8	18
Tissue	Brain			Heart muscle		
Uncoupling reagent	None	Salicylate	DNP	None	Salicylate	DNP
Amino acids	76	72	57	66	62	56
Glutamic	43	44	23	21	20	23
Alanine	8	7	14	19	1	1
Aspartic	16	15	5	3	22	19
Organic acids	16	23	33	21	21	21
Lactic acid	2	2	4	6	8	2

Table 4. *Effects of 5 mM-salicylate and 0·5 mM-2:4-dinitrophenol on transaminase activities in rat serum*

Results are expressed as mean percentage inhibitions (±S.E.M.) of the values found in the absence of salicylate or DNP. The results have been analysed by the *t* test, Bessel's correction for small samples being used, and values of *P* are included. The number of observations are given in parentheses.

Transaminase activity	Salicylate	DNP
Glutamic–pyruvic	40±1·4 (8) $P < 0.01$	0·6±0·5 (6) $P > 0.9$
Glutamic–oxaloacetic	11±0·4 (8) $P < 0.05$	0·4±0·8 (6) $P > 0.9$

The occurrence of ^{14}C in citric acid, succinic acid, malic acid and fumaric acid (and in α-oxoglutaric acid in brain) is evidence that the pyruvate carbon atoms entered the tricarboxylic acid cycle. A considerable proportion of the radioactivity was found in amino acids (glutamic and aspartic) which are formed from α-oxoglutarate and oxaloacetate by transamination reactions. The presence of the isotope in methionine and leucine demonstrates the ability of the isolated rat tissues to synthesize amino acids which are generally considered to be essential constituents in the diet. The incorporation of radioactivity into glucose in liver, brain and kidney indicates the presence of synthetic pathways and these tissues also incorporated trace amounts of ^{14}C into substances which were presumptively identified from their chromatographic positions as phosphoenolpyruvate and phosphoglycerate.

In liver, kidney and brain both uncoupling reagents decreased the incorporation of ^{14}C into glucose; radioactivity, when present, was virtually restricted to the amino and organic acids. A difference was observed between the effects of salicylate and DNP on the distribution of ^{14}C between the amino and organic acid fractions. Salicylate produced proportionately more incorporation of radioactivity into the amino acids than into the organic acids whereas DNP caused the reverse effect (Table 3). Glutamic acid and lactic acid were the substances principally involved in these redistributions and in liver and kidney salicylate caused a greater percentage incorporation of ^{14}C into glutamic acid than occurred in the corresponding control experiments. This effect on glutamic acid was not found with heart muscle, in which both uncoupling reagents produced similar effects in decreasing the accumulation of radioactivity in alanine and increasing it in aspartic acid.

Effects of salicylate and 2:4-dinitrophenol on rat serum transaminase activities. The results in Table 4 show that 5 mM-salicylate caused a significant inhibition *in vitro* of the serum glutamic–pyruvic-transaminase activity and a smaller, but still significant, effect on the serum glutamic–oxaloacetic transaminase activity; 0·5 mM-DNP had no effect on either enzyme activity.

Incorporation of ^{14}C *from* L-[^{14}C]*glutamate into the soluble intermediates of isolated rat kidney.* The results in Table 5 show the amounts of ^{14}C incorporated into the soluble intermediates of kidney in the presence or the absence of salicylate and DNP.

In the absence of the uncoupling reagents radioactivity was found in γ-aminobutyric acid, showing that decarboxylation of the glutamic acid had occurred. The presence of ^{14}C in fumaric acid and

Table 5. *Metabolism of* L-[^{14}C]*glutamic acid by rat kidney in the presence or the absence of* 5 mM-*salicylate or* 0·5 mM-2:4-*dinitrophenol*

Details were as given for Table 3 except that each flask contained 8·5 mg. dry wt. of kidney, and 4·5 μc (2·6 μmoles) of labelled glutamic acid was used as substrate.

Uncoupling reagent ...	None	Salicylate	DNP
Glucose	32	0	0
Serine	14	0	0
γ-Aminobutyric acid	67	85	34
Aspartic acid	210	125	173
Fumaric acid	3	14	10
Malic acid	37	49	80
Lactic acid	19	44	53
Unidentified compounds	35	67	45
Total in soluble fraction	415	390	395
Residual glutamic acid	4900	5300	5300

malic acid is evidence that the glutamate carbon atoms entered the tricarboxylic acid cycle and the radioactive aspartic acid was presumably derived by transamination from oxaloacetate. The incorporation of the isotope into lactic acid, serine and glucose indicated the presence of synthetic pathways. The main effects of salicylate and DNP were to prevent the appearance of radiocarbon in serine and glucose and to increase the incorporation of the isotope into the organic acids. DNP caused a decreased appearance of ^{14}C in γ-aminobutyric acid whereas salicylate had the reverse effect. Both substances had little effect either on the total incorporation of ^{14}C into the soluble intermediates or on the amount of radioactivity recovered in the residual glutamic acid.

DISCUSSION

Chopped preparations of rat tissues incorporate radioactivity from [3-^{14}C]pyruvate into alanine and lactic acid which are derived directly from the pyruvate by transamination and dehydrogenation reactions. The occurrence of ^{14}C in citric acid, succinic acid, malic acid and fumaric acid shows that the pyruvate carbon atoms entered the tricarboxylic acid cycle, and a substantial proportion of the radioactivity was found in glutamic acid and aspartic acid, which are formed from α-oxoglutaric acid and oxaloacetic acid by transamination reactions. The presence of synthetic pathways is indicated by the incorporation of ^{14}C into glucose by liver, kidney and brain; kidney also formed labelled glucose and serine when incubated with L-[^{14}C]glutamate. Katz & Chaikoff (1955) have shown that isolated rat-liver slices incubated with labelled acetate incorporate radioactivity into glucose.

It must be emphasized that the present experiments provide information only about the effects of the uncoupling reagents on the transfer of radiocarbon from the labelled substrates to the soluble intermediates. No attempt has been made to measure the pool size and hence the specific activity of each intermediate and it is therefore not possible to decide if any interference with either the production or utilization of unlabelled material has contributed to the observed effects.

Salicylate produced a number of effects on the incorporation of radioactivity from the labelled pyruvate into the soluble intermediates. Some of these, such as the decreased total ^{14}C incorporation (Table 1) and the decreased appearance of radiocarbon in glucose (Table 2), were also caused by DNP and were therefore related to an uncoupling action on oxidative-phosphorylation processes. However, in the liver and kidney preparations, salicylate, but not DNP, caused an increased accumulation of radioactivity in glutamic acid which exceeded the values found in the absence of the uncoupling reagents (Table 3). The incorporation of ^{14}C into glutamic acid in brain in the presence of salicylate was also greater than that which occurred in the presence of DNP. These results suggested that salicylate exerted an effect on glutamic acid metabolism distinct from its uncoupling action.

The principal route for the incorporation of ^{14}C from the labelled pyruvate into glutamic acid is probably via acetyl-coenzyme A and α-oxoglutarate. The more important pathways involved in the further metabolism of glutamic acid include protein synthesis, decarboxylation to γ-aminobutyric acid and various transamination reactions (Meister, 1957). Both salicylate and DNP inhibit the incorporation of ^{14}C-labelled amino acids into protein in isolated tissues (Siekevitz, 1952; Manchester, Randle & Smith, 1958) and the present results (Table 2) show that both uncoupling reagents decreased the formation of radioactive γ-aminobutyric acid from the labelled pyruvate. However, 5 mM-salicylate, but not 0·5 mM-DNP, was found to inhibit the activities of the two transaminases involving glutamic acid which are present in rat serum. The effect of salicylate on the serum glutamic–pyruvic-transaminase activity was particularly pronounced and has been confirmed in other work (Steggle, Huggins & Smith, 1961). Manso, Taranta & Nydick (1956) have reported that 0·5–3 mM-salicylate has no effect *in vitro* on either of the two transaminase activities in human serum.

Inhibition of transaminase reactions involving glutamic acid may therefore explain, at least in part, the relative accumulation of radioactivity in the glutamic acid when isolated preparations of rat liver and kidney are incubated with [3-^{14}C]-

pyruvate in the presence of 5 mM-salicylate. An attempt to obtain more direct evidence relating to this hypothesis was made by incubating isolated rat kidney with L-[^{14}C]glutamic acid in the presence or the absence of salicylate or DNP. However, there was little, if any, difference between the effects of the uncoupling reagents. The main changes concerned an inhibition of synthetic reactions, e.g. the incorporation of radioactivity from the labelled glutamate into serine and glucose, and an increased appearance of the isotope into lactic acid, malic acid and fumaric acid. The transfer of ^{14}C from the labelled substrate to γ-aminobutyric acid was decreased in the presence of DNP but salicylate caused a slight increase. No difference between the effects of salicylate and DNP on the amount of residual [^{14}C]glutamic acid was observed. Thus the metabolism of glutamate appeared to have a different sensitivity to salicylate, depending on whether the glutamate was formed from pyruvate or was supplied externally.

SUMMARY

1. The effects of 5 mM-salicylate and 0·5 mM-2:4-dinitrophenol on the incorporation of ^{14}C from [3-^{14}C]pyruvate and L-[^{14}C]glutamate into the soluble intermediates of chopped rat tissues has been studied.

2. In the absence of the uncoupling reagents, the tissue preparations incorporated the isotope into substances known to be involved in the tricarboxylic acid cycle, in transamination and in synthetic reactions.

3. Salicylate and dinitrophenol produced similar effects on the distribution of radioactivity in the soluble intermediates which appear to be explicable in terms of their uncoupling action on oxidative-phosphorylation reactions.

4. Salicylate, but not dinitrophenol, caused an increased incorporation of ^{14}C from the [3-^{14}C]-pyruvate into glutamic acid in liver and kidney and also inhibited the activities of rat-serum glutamic–pyruvic and glutamic–oxaloacetic transaminases.

5. Salicylate and dinitrophenol had similar effects on the distribution of ^{14}C from L-[^{14}C]-glutamate in chopped rat kidney.

6. Some implications of these results are discussed.

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Uncoupling Reagents and Metabolism

4. EFFECTS OF SALICYLATE ADMINISTRATION IN THE RAT ON THE INCORPORATION OF ^{14}C FROM LABELLED GLUCOSE AND ACETATE INTO THE SOLUBLE INTERMEDIATES OF ISOLATED LIVER AND BRAIN

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The incubation of salicylate with isolated rat tissues produces effects on the total incorporation and subsequent distribution of ^{14}C from [^{14}C]-glucose and [2-^{14}C]acetate in the soluble intermediates, which are explicable in terms of an uncoupling action on oxidative-phosphorylation processes (Smith & Moses, 1960). In the present study, isolated liver and brain preparations were incubated with labelled glucose or acetate after the animals had received either single or repeated

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injections of salicylate. The work was undertaken to determine if the acute or chronic administration of salicylate to the intact rat produced effects on the incorporation of radioactivity into the soluble intermediates of the isolated tissues similar to those resulting from incubation with the uncoupling reagent *in vitro*. In addition, the effect of adding salicylate to the incubation media containing the tissue from the rats previously injected with salicylate was also investigated.

EXPERIMENTAL

Male rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet no. 41, were given either a single intraperitoneal injection of 1 ml. of sodium salicylate solution or received 1 ml. injections of the salicylate daily at 9 a.m., for periods up to 92 days. The single injections contained either 928 mg. of sodium salicylate/kg. body wt. ('lethal dose'), the animals being killed by decapitation 90 min. after the injection, or 464 mg. of sodium salicylate/kg. body wt. ('half-lethal dose'), when the rats were killed 3 hr. after the injection. The daily injections contained 543 mg./kg. body wt. ('chronic dose'), the rats being killed 3 hr. after the last injection. The methods used for the preparation of the chopped liver and brain, for the incubation with [^{14}C]glucose and [2-^{14}C]acetate in the presence or the absence of 5 mM-salicylate, and for the chromatographic separation and measurement of radioactivity in the soluble intermediates, were those described by Smith & Moses (1960). The ^{14}C-labelled materials were obtained from The Radiochemical Centre, Amersham, Bucks.

A sample of blood was obtained from each salicylate-treated animal at the time of killing, and analysed for salicylate by the method of Trinder (1954).

RESULTS

The radioactivity measured was that present in the soluble intermediates of the cell extract and medium. The total incorporation of ^{14}C from the labelled glucose or acetate into all the soluble intermediates is given as counts/min./unit wt. of tissue. However, some variation occurs in the metabolic activities of tissue preparations from different animals. The distribution of the radiocarbon among the individual soluble intermediates has therefore been expressed as percentage values of the total ^{14}C incorporation, rather than as absolute counts.

Liver

Metabolism of [^{14}C]*glucose*. The results in Table 1 show that the salicylate injections did not affect the total incorporation of ^{14}C from the labelled glucose. The main effects on the subsequent distribution of the isotope among the soluble intermediates involved maltose, amino acids and lactate. Single injections of either the lethal or half-lethal dose of salicylate caused a substantial decrease of ^{14}C in the maltose, whereas repeated injections of the chronic doses produced the reverse effect. The incorporation of the radiocarbon into glutamic acid was considerably enhanced by all types of salicylate injection, although the effect gradually diminished during the course of repeated injections. In contrast, the formation of labelled alanine was decreased by the salicylate injections, the inhibition becoming more pronounced during the continued administration of the chronic doses. The accumulation of the isotope in lactate was greatly increased by both single and repeated injections of salicylate, and decrease of radioactivity in the sugar phosphates and nucleotides occurred. When salicylate was added to the incubation media, its main effects were to decrease the incorporation of ^{14}C into lactate and to prevent the decrease of radioactivity in the sugar phosphates and nucleotides noted above.

Metabolism of [2-^{14}C]*acetate*. The total incorporation of ^{14}C from labelled acetate was not altered by either single or repeated injections of salicylate, but was considerably decreased by the addition of salicylate to the incubation media (Table 2). The administration of either the lethal or half-lethal dose of salicylate decreased the incorporation of radioactivity into aspartic acid and the reverse effect occurred during repeated injections of the chronic dose. All the salicylate injections stimulated the production of labelled γ-aminobutyric acid, but its formation was depressed in the presence of added salicylate in the incubation medium, which also caused an increased accumulation of ^{14}C into lactic acid.

Brain

Metabolism of [^{14}C]*glucose*. The results in Table 3 show that the administration of salicylate to the whole animal produced no effects in the total incorporation of ^{14}C and that the single injections caused little change in the subsequent distribution of the isotope among the soluble intermediates. Repeated injections of the chronic dose somewhat decreased the radioactivity in glutamic acid, γ-aminobutyric acid and alanine and increased it in glutamine and succinic acid and malic acid. The presence of added salicylate in the incubation medium caused some increase in the ^{14}C-labelled citrate.

Metabolism of [2-^{14}C]*acetate*. The total incorporation of ^{14}C was not decreased unless added salicylate was present in the incubation medium (Table 4). The salicylate injections caused an increased incorporation of the isotope into aspartic acid accompanied by an inhibition of the formation of labelled glutamic acid. When salicylate was added to the incubation medium the incorporation of radioactivity into lactic acid was increased in the brain of those animals receiving the chronic dose of salicylate for periods up to 43 days.

Table 1. *Effect of salicylate administration* in vivo *on the metabolism of* [^{14}C]*glucose by rat liver* in vitro *in the presence or the absence of added salicylate*

Each flask contained about 30 mg. dry wt. of tissue in a total vol. of 1 ml. +, Presence of 5 mM-salicylate in the medium; −, absence of salicylate. The tissue was incubated with 10 μc of labelled substrate for 3 hr. at 37° in air. The ^{14}C present in each compound is expressed as a percentage of the total ^{14}C incorporated from the labelled substrate into the sum of all the soluble intermediates; ^{14}C in the residual substrate is excluded from all calculations. Values for the total incorporation of ^{14}C are expressed as 10^{-3} counts/min./25 mg. dry wt. of tissue. UDPG, Uridine diphosphoglucose.

Salicylate injections ...	None (control)		Half-lethal		Lethal		Chronic (8 days)		Chronic (16 days)		Chronic (29 days)		Chronic (43 days)		Chronic (92 days)	
Blood salicylate concn. at time of death (mg./100 ml.)	—		72		144		41		74		41		60		37	
Salicylate in the medium	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Aspartic acid	15	21	12	13	16	16	12	13	12	9·7	8·5	8·0	6·0	6·1	8·5	9·3
Glutamic acid	0·9	1·0	33	15	21	13	14	14	6·3	6·2	2·7	2·7	0·8	1·8	2·0	1·2
Alanine	26	25	12	19	21	21	7·5	9·0	5·3	15	7·8	8·9	3·6	5·0	11	12
Glutamine	0	0	0	0	0	3·6	0·2	0·2	0	0	0·3	0	0·2	0	0	0
γ-Aminobutyric acid	0	0	0	0	2·1	2·9	0	0	0	0	0·1	0	0	0	1·6	1·2
Lactic acid	1·2	6·7	27	6·6	12	8·9	12	3·8	18	4·9	26	8·1	27	9·5	12	14
Malic + succinic + fumaric acid	0	0	0	0	0·1	0·3	0	0	0·3	0·3	0·2	0·1	2·0	3·2	2·0	0
Citric acid	0	0	0	0·1	0	0·1	0	0	0·4	0·4	0	0	0·1	0	0	0
Total in:																
Sugar phosphates + UDPG + nucleotides	24	7·7	7·9	10	16	16	12	13	13	14	11	9·9	8·8	9·9	15	13
Maltose	10	9·1	0·5	3·9	0·3	1·0	26	27	21	28	35	40	37	43	37	43
Amino acids	52	71	64	64	68	64	45	43	31	44	28	38	16	15	29	25
Organic acids	1·5	6·7	27	6·7	16	17	12	3·8	19	6·7	26	8·3	35	26	19	17
Other substances	13	5·4	0·5	15	1·5	2·1	4·8	13	15	8·1	0·3	3·6	3·0	7·5	0	0
Total ^{14}C in soluble extract	150	99	140	100	160	170	190	190	190	190	100	110	110	99	130	120

Table 2. *Effect of salicylate administration* in vivo *on the metabolism of* [2-^{14}C]*acetate by rat liver* in vitro *in the presence or the absence of added salicylate*

Details were as given in Table 1.

Salicylate injections ...	None (control)		Half-lethal		Lethal		Chronic (8 days)		Chronic (16 days)		Chronic (29 days)		Chronic (43 days)		Chronic (92 days)	
Salicylate in the medium	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Aspartic acid	7·8	6·5	5·9	2·9	5·0	4·2	12	3·9	11	3·5	11	5·3	9·2	2·6	9·7	6·0
Glutamic acid	12	1·4	9·9	14	12	22	12	13	9·9	8·3	14	12	9·7	8·3	20	17
Alanine	4·3	7·8	4·9	5·6	5·6	7·6	3·5	3·3	2·7	3·7	4·1	2·4	4·8	5·2	3·2	6·0
Glutamine	4·3	0	1·9	4·6	3·9	8·4	2·5	4·0	2·9	4·6	6·5	2·6	7·0	3·2	4·8	3·0
γ-Aminobutyric acid	0·7	26	8·0	3·4	4·6	5·4	2·6	1·5	2·2	1·3	5·2	0·3	4·2	1·2	7·9	3·0
Glucose*	22	10	25	9·1	27	6·1	9·2	2·0	22	4·0	8·2	0·2	10·8	3·8	8·6	3·4
Lactic acid	25	4·7	18	29	17	13	11	25	27	40	22	45	23	55	26	14
Malic + succinic + fumaric acid	0·6	15	1·5	4·0	5·4	3·3	3·4	1·5	3·2	3·9	2·9	5·4	2·4	5·7	2·5	4·5
Citric acid	0·1	0·6	0·1	0·1	0·1	0·3	0·1	0·1	0·2	0·6	0·2	0·2	0·1	0·2	0·1	0·7
Total in:																
Amino acids	62	62	61	54	72	60	66	54	63	45	66	37	58	33	59	45
Organic acids	26	27	26	36	24	24	17	29	33	47	27	52	25	61	29	20
Other substances	13	11	12	11	4·5	16	17	17	3·9	8·2	6·8	11	18	5·9	13	35
Total ^{14}C in soluble extract	160	12	160	89	250	120	260	110	200	120	250	90	220	88	270	52

* The glucose spot contained small amounts of serine and glycine.

Table 3. *Effect of salicylate administration* in vivo *on the metabolism of* [^{14}C]*glucose by rat brain* in vitro *in the presence or the absence of added salicylate*

Details were as given in Table 1 except that each flask contained about 9·0 mg. dry wt. of brain.

Salicylate injections ...	None (control)		Half-lethal		Lethal		Chronic (8 days)		Chronic (16 days)		Chronic (29 days)		Chronic (43 days)		Chronic (92 days)	
Salicylate in the medium	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Aspartic acid	24	21	28	28	27	30	27	19	26	25	23	17	25	29	23	17
Glutamic acid	19	26	14	19	20	22	12	15	12	18	13	22	14	20	12	19
Alanine	26	17	18	12	23	12	13	10	12	8·1	10	8·4	12	15	14	11
Glutamine	0·7	0	0	0	0	0	0·1	0·1	0	0	8·9	12	7·7	10	9·5	11
γ-Aminobutyric acid	13	7·7	8·9	7·3	15	11	7·0	6·1	7·1	5·7	9·4	6·7	9·3	7·6	9·5	8·4
Lactic acid	4·5	2·7	13	3·5	0	3·2	3·9	1·7	7·5	0	7·6	3·3	9·9	1·7	0·4	4·9
Malic + succinic + fumaric acid	3·0	3·1	3·3	3·2	3·5	5·1	6·9	8·8	6·9	8·0	5·5	5·1	4·9	3·4	8·3	4·9
Citric acid	6·9	14	6·0	9·3	4·7	8·6	4·8	11	4·0	9·9	5·7	12	4·0	3·5	7·6	14
Total in:																
Sugar phosphates + UDPG + nucleotides	4·3	2·2	2·6	3·9	2·9	2·7	2·8	3·6	5·3	5·4	3·0	2·0	3·2	1·7	3·2	2·5
Amino acids	82	77	74	73	85	75	65	54	64	62	71	69	72	86	72	68
Organic acids	14	20	22	17	12	20	18	27	21	22	22	24	19	8·5	16	23
Other substances	0·7	1·2	1·3	6·1	0·3	2·5	14	5·0	10	11	4·5	5·4	6·2	3·7	8·5	6·5
Total ^{14}C in soluble extract	900	1100	1500	1500	1300	1000	1300	1400	1400	1200	1100	1100	1100	1000	950	990

Table 4. *Effect of salicylate administration* in vivo *on the metabolism of* [2-^{14}C]*acetate by rat brain* in vitro

Table 4. *Effect of salicylate administration* in vivo *on the metabolism of* [2-^{14}C]*acetate by rat brain* in vitro *in the presence or the absence of added salicylate*

Details were as given in Table 1 except that each flask contained about 9·0 mg. dry wt. of brain.

Salicylate injections ...	None (control)		Half-lethal		Lethal		Chronic (8 days)		Chronic (16 days)		Chronic (29 days)		Chronic (43 days)		Chronic (92 days)	
Salicylate in the medium	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Aspartic acid	25	16	29	21	39	30	35	33	37	34	33	21	38	29	39	33
Glutamic acid	31	22	22	16	28	18	26	21	22	23	28	27	28	36	26	25
Alanine	3·2	0	2·6	5·3	0·4	0	0·2	6·4	4·1	0	2·3	0	0·3	0	1·6	0
Glutamine	0·2	0	0	0	0	0	1·2	0	0·1	0	0·7	0	0·6	0	0	0
γ-Aminobutyric acid	13	14	9·5	17	10	5·0	7·7	4·7	7·0	3·3	12	0	13	15	11	7·9
Lactic acid	6·1	7·1	13	9·6	0	0	11	22	0	14	3·7	28	1·8	3·9	0	0
Malic + succinic + fumaric acid	5·0	12	3·1	4·3	5·3	34	10	2·5	11	11	3·1	11	5·4	14	9·2	9·1
Citric acid	7·3	7·6	4·6	3·9	3·3	6·6	0·2	9·2	4·9	10	3·8	7·7	6·0	1·9	7·3	12
Total in:																
Amino acids	72	52	66	66	78	53	70	65	73	59	78	48	84	80	77	65
Organic acids	18	26	25	19	21	42	30	34	22	41	17	52	13	19	16	21
Other substances	9·0	21	9·6	15	1·4	5·7	0·7	1·6	5·1	0	4·9	0	2·8	0·6	6·9	14
Total ^{14}C in soluble extract	130	40	240	64	83	30	130	25	200	23	180	28	190	31	180	55

DISCUSSION

The single injection of either the lethal or the half-lethal dose of salicylate caused similar effects on the metabolism of labelled glucose and acetate by liver and brain, despite the difference in blood concentrations of salicylate produced in the animals. The most prominent changes resulting from the single injections were concerned with the distribution of radioactivity from the [^{14}C]glucose in the soluble intermediates of liver. The incorporation of ^{14}C into maltose, sugar phosphates, nucleotides and alanine was decreased whereas that into glutamic acid and lactic acid was increased. These changes, although more pronounced, were similar to those produced by the incubation of salicylate *in vitro* with liver from untreated rats metabolizing [^{14}C]glucose (Smith & Moses, 1960). An exception was the greatly increased accumulation of radioactivity in glutamic acid observed in the injected animals. However, Huggins, Smith & Moses (1961) have shown that salicylate inhibits glutamic–pyruvic-transaminase activity in rat serum. It also caused an increased incorporation of ^{14}C into glutamic acid in liver preparations (from untreated rats) incubated with [3-^{14}C]pyruvate. Such an inhibition of the transaminase activity could account for the increased amounts of labelled glutamic acid and the decreased formation of labelled alanine from [^{14}C]glucose in the liver of the rats injected with either single or repeated doses of salicylate. This effect may not have been detectable with the liver from untreated rats, since only traces of radioactivity from [^{14}C]glucose appeared in glutamic acid.

A marked difference between the effects of the single injections and of repeated injections of the chronic dose occurred with respect to the incorporation of radiocarbon from [^{14}C]glucose into maltose by the liver. The administration of the lethal or half-lethal dose caused a substantial decrease of radioactivity in the maltose, whereas the repeated injections caused a greatly increased accumulation of isotope in the disaccharide. Prolonged administration of salicylate has been reported to cause increased blood concentrations of 17-hydroxycorticosteroids in the rat (van Cauwenberge, 1955), and Moses & Smith (1961) have shown that incubation of rat liver metabolizing [^{14}C]glucose with cortisol causes an increase in radioactivity in maltose. These observations suggest that the increased incorporation of radioactivity into maltose from the [^{14}C]glucose by the liver of rats which received prolonged treatment with the chronic dose of salicylate may result from an enhanced adrenal-cortical activity in the animals.

In contrast with the results with liver metabolizing [^{14}C]glucose, the injections of salicylate

had relatively little effect on either the total incorporation or the subsequent distribution of ^{14}C in liver or brain incubated with [2-^{14}C]acetate, or in brain with [^{14}C]glucose. The decrease in the total incorporation of ^{14}C from the labelled acetate in the liver and brain, and the increased incorporation of radioactivity from the labelled glucose by brain, which occurred in the tissues of untreated rats incubated with 5 mM-salicylate (Smith & Moses, 1960), was not observed in similar preparations from the injected animals unless 5 mM-salicylate was also included in the incubation media. The probable explanation for these differences is the inevitable loss of salicylate during the preparation of the isolated tissues from the rats injected with salicylate, particularly as many of the effects could again be elicited by adding salicylate to the incubation medium. However, only those effects of salicylate *in vitro* which appeared to be associated with an uncoupling action behave in this way. This suggests that the isolation and preparation of tissue from salicylate-treated animals was sufficient to remove salicylate from sites where it could uncouple oxidative phosphorylation, but not from other locations such as the glutamic–pyruvic-transaminase system. Thus reactions dependent on oxidative phosphorylation (e.g. the uptake of [2-^{14}C]acetate) operated in an uninhibited manner when the tissue was removed from the animal and washed, but were still sensitive to the presence of added salicylate in the incubation medium even after prolonged administration of salicylate to the intact animal. On the other hand, the formation of labelled alanine was depressed even after the tissue had been washed, and no further depression was obtained by the addition of extra salicylate to the incubation mixture.

SUMMARY

1. The effects of single and repeated injections of salicylate in the rat on the incorporation of ^{14}C from labelled substrates into the soluble intermediates of isolated liver and brain preparations have been studied.

2. The total incorporation of radioactivity from [^{14}C]glucose or [2-^{14}C]acetate in liver and brain was not altered by the injections.

3. The distribution of ^{14}C from [^{14}C]glucose in liver was affected by both single and repeated injections of salicylate. The main changes were a decreased incorporation of radiocarbon into alanine, sugar phosphates and nucleotides and an increased incorporation into glutamic acid and lactic acid. The accumulation of ^{14}C into maltose was decreased by the single injections and increased by the repeated injections.

4. Some implications of these results are discussed.

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The Distribution of Readily Reducible Disulphide Bonds in Wool

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Elsworth & Phillips (1938, 1941) observed that only about half the disulphide groups in wool were broken by treatment with solutions of bisulphite at pH 5·0 and they attributed this to differences in the reactivity of the cystine residues. On the basis of this work and the demonstration by Patterson, Geiger, Mizell & Harris (1941) that reduction with mercaptoacetate gives similar results, Middlebrook & Phillips (1942) suggested that the extent of reaction was not related to the nature of the reducing agent. They postulated the existence of four classes of disulphide bonds, namely those belonging to the A, B, C and D fractions, the first two being reducible at pH 5·0 by solutions of bisulphite or mercaptoacetate. These findings have since been interpreted as being due to

three possible causes. First, to variations in the morphological localization of the disulphides within the fibre; secondly to the effects of neighbouring side-chain groups (either adjacent or brought close by folding); and finally to differences in reactivity of intra- and inter-chain disulphide bonds.

The present study is concerned with assessing the importance of the above-mentioned factors in limiting disulphide reactivity. In particular, information was sought about the distribution of the easily reducible disulphide bonds in various soluble wool proteins. This was done by reducing the wool at pH 5·0 with mercaptoacetate, followed by labelling the sulphydryl groups with [2-^{14}C]-iodoacetate. Various labelled protein components were then isolated by fractional extraction.

MATERIALS

Wool. Solvent-degreased Merino 64's wool top (moisture content 11·5%) was used in these experiments.

Chemicals. Analytical-grade reagents were used throughout.

[2-^{14}C]*Iodoacetic acid.* [2-^{14}C]Bromoacetic acid (specific activity 3 mc/m-mole) was purchased from The Radiochemical Centre, Amersham, Bucks. A solution of 1 mc, made up to 100 ml. with water, was prepared and stored in the frozen state. To 1 ml. (10 μc) was added solid unlabelled carrier (bromoacetic acid) in amounts ranging from 250 to 2000 μmoles, depending on the activity required.

The bromoacetic acid was extracted from the solution with three lots of 5 ml. of anaesthetic ethyl ether, the combined extracts were evaporated to dryness and the bromoacetic acid was then dissolved in 5 ml. of acetone. To this solution was added the theoretical amount of sodium iodide in 5 ml. of acetone for conversion into iodoacetic acid. After the reaction had proceeded for 5 min. the precipitate of sodium bromide was removed by centrifuging and washed once with 5 ml. of acetone.

The combined supernatant and washing was concentrated almost to dryness under a stream of air and dissolved in about 20 ml. of water. This solution was neutralized with N-sodium hydroxide and buffered at pH 9·3 by the addition of 0·8 g. of sodium borate.

The solution was used within a few hours of preparation. The recovery of radioactivity indicated yields of over 90%.

METHODS

Reduction of wool at pH 5·0 *and alkylation with* [2-^{14}C]*iodoacetate*

Sodium mercaptoacetate (25 ml., 1 M, pH 5·0) was added to wool (1 g.), the solution was de-aereated by evacuation and the reaction allowed to proceed for 18 hr. at about 20° for complete reduction of the (A+B) fraction or for 2 hr. at 10° for partial reduction. The reduced wool was thoroughly washed with distilled water, dried by squeezing between filter papers, placed in 25 ml. of [2-^{14}C]iodoacetate solution, the mixture was de-aerated and the pH maintained at 9·0 until the nitroprusside test for SH groups was negative. The wool was then washed and dried.

The amount of reduction in the initial treatment at pH 5·0 was determined indirectly by measuring the amount of radioactive iodoacetate remaining in the solution after alkylation and by direct measurement of residual disulphide in the alkylated protein, by the amperometric method of Leach (1959, 1960). These estimates are subject to some error both because of possible side reactions between the wool and iodoacetate (Gundlach, Moore & Stein, 1959) and because of mixed disulphide formation (Human & Springell, 1959).

Extraction of labelled wool

In extracting the proteins from labelled wool two procedures were used, which were based on the earlier work of Gillespie & Lennox (1955) and Gillespie (1960). These two methods describe a multiple short (M) and a single long (L) extraction respectively.

Method L. The labelled wool derived from 1 g. was extracted with 100 ml. of 0·1 M-potassium mercaptoacetate for 2 hr. at 50° (initial pH 11·0, final pH 10·5). The protein solution was filtered, treated with 30 ml. of a solution containing 4 g. of unlabelled iodoacetic acid and 7 g. of sodium borate and the pH maintained between 8·5 and 9·0 until the nitroprusside test became negative (Fraction L1). In some experiments the extraction was terminated here, but in others the residue was extracted for 22 hr. at 40° with 100 ml. of 0·1 M-potassium mercaptoacetate containing 8 M-urea at an initial pH of 11·0. The soluble proteins (Fraction L2) derived from this extraction were alkylated. The residue from each type of extraction (Fractions L1R and L2R) were also alkylated and then dialysed together with the alkylating solution and freeze-dried.

Method M. Labelled wool (from 10 g.) was extracted five times successively with 300 ml. lots of 0·1 M-potassium mercaptoacetate, each for 20 min. at 50°. In preparation 5 (Table 2) only the first extract was prepared and its residue collected. In preparation 4 (Table 2), the first five extracts, with final pH values ranging from 9·7 to 10·4, were pooled and precipitated at pH 5·0 with acetic acid, and the precipitate was collected by centrifuging. This material (Fraction M1) was dissolved in 300 ml. of 0·1 M-mercaptoacetate and the SH groups were alkylated with 12 g. of unlabelled iodoacetic acid and 21 g. of sodium borate.

The residue was extracted for a further 20 min. at 50° with 0·1 M-mercaptoacetate (initial pH 12·2, final pH 11·4) and the extracted protein (Fraction M2) was alkylated. The residue was next extracted as described in method L with 300 ml. of urea–mercaptoacetate solution and the soluble proteins (Fraction M3) were alkylated as in Fraction M1. The final residue (Fraction M3R) was also alkylated and, after washing with ethanol, was dried *in vacuo* over phosphorus pentoxide; some protein (representing about 2% of the original wool) which dissolved in the alkylating solution was isolated by dialysis and freeze-drying (Fraction M4).

Fractionation into proteins with high- and low-sulphur contents

The various alkylated soluble fractions were dialysed against running tap water for 18 hr. and then precipitated by dialysis against acetate buffer (pH 4·1, *I* 0·1) as de-

scribed by Gillespie (1960). The precipitate of low-sulphur protein was collected by filtration, washed with water and dissolved in 15 ml. of dilute ammonia solution. The supernatant, containing the high-sulphur protein, was dialysed against running tap water overnight, freeze-dried and dissolved in 15 ml. of water.

Reduction, extraction and fractionation of unlabelled wool

For comparative purposes the above extractions and fractionations were repeated on untreated wool and on wool which had been reduced at pH 5·0 and alkylated with unlabelled iodoacetate. The relation between pH and solubility in 0·1 M-mercaptoacetate was studied on these wool samples.

Isolation and specific-activity determination of S-carboxymethylcysteine

Freeze-dried labelled protein (up to 300 mg.) was hydrolysed with 5 ml. of constant-boiling hydrochloric acid for 20 hr. at 105°. The hydrolysate was filtered, dried and chromatographed by a modification of the Hirs, Moore & Stein (1954) procedure. Dowex-2 (acetate form; X 8) was used in 2 cm. × 50 cm. columns with 1 N-hydrochloric acid as the sole eluent. Under these conditions the *S*-carboxymethylcysteine was eluted after 230 ± 18 ml., well separated from its nearest neighbour, aspartic acid. The *S*-carboxymethylcysteine content was determined on small portions (25–100 μl.) of each chromatographic fraction by the ninhydrin method (Moore & Stein, 1954). The total *S*-carboxymethylcysteine content was then calculated, a colour factor of 1·28 being used (R. J. Rowlands & D. H. Simmonds, unpublished work).

The pooled chromatographic fractions from each run were freeze-dried, the freeze-dried fractions were made up to 10 ml. with water and the radioactivity was measured in an ionization chamber on a 100 cm.^{2} sample pan with a model 6000 Dynacon Electrometer (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Radioactivity in terms of μC was calculated in each sample from the ionization current (Springell, 1960), and the specific activity was then expressed as μC/m-mole of *S*-carboxymethylcysteine.

Reactions for increasing disulphide fission at pH 5·0

With bisulphite. Wool (1 g.) was caused to react at 20° for 42 hr. with a solution containing 20 m-moles of sodium bisulphite and 1 m-mole of neohydrin (3-chloromercuri-2-methoxypropylurea) in 100 ml. of acetate buffer (pH 5·0, *I* 0·2).

With mercaptoacetate. Wool (0·5 g.) was caused to react under nitrogen at 20° with four successive lots of 1 l. of 1 M-sodium mercaptoacetate, over a total period of 144 hr. The wool was degassed after each change and was finally caused to react under nitrogen with iodoacetate overnight at 20°.

The residual disulphide in each preparation was estimated by the method of Leach (1959, 1960).

RESULTS

Effect on wool fractionation of reduction at pH 5·0 *and subsequent alkylation.* Fig. 1 shows that the curves relating pH to percentage extraction by mercaptoacetate solution are similar for untreated and for reduced and alkylated wool. The most notable differences are that the latter was solubilized at slightly lower pH values and that the yield of high-sulphur protein was decreased.

Similarly (Table 1) the sulphur contents of the fractions obtained from both sources by precipitation at pH 4·1 are alike. It is clear that the separation of high- and low-sulphur fractions is largely unaffected by the pretreatment.

Proportion of disulphide residues reduced at pH 5·0 *in various fractions.* Table 2 shows that the specific activities of the *S*-carboxymethylcysteine, and hence the proportion of disulphide groups reduced at pH 5·0, are the same for the high- and low-sulphur components of each fraction. The fractions themselves differ slightly one from the other in their specific activities, but all the values

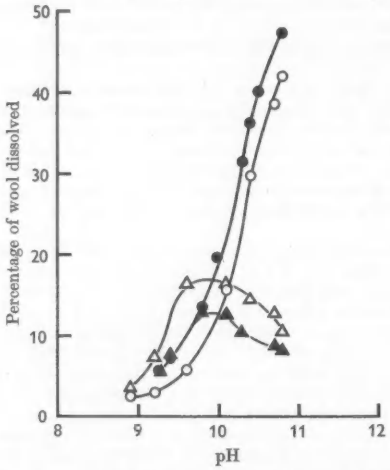

Fig. 1. Comparison of the yields of protein extracted by alkaline mercaptoacetate from reduced and alkylated wool and from untreated wool as a function of the final pH of extraction. Wool was reduced at pH 5·0 with 1 M-mercaptoacetate for 18 hr. at 20° and alkylated with unlabelled iodoacetate (see Methods). Portions (1 g.) of this and of a control wool were extracted with portions (30 ml.) of 0·1 M-mercaptoacetate over the range pH 9–11 for 3 hr. at 40°. The extracts were coupled with iodoacetate, dialysed and the extinction at 277 mμ was measured. They were then precipitated at pH 4·1 and the supernatants (high-sulphur protein) separated by centrifuging and their extinctions also measured ($E^{1\%}_{1\,cm.}$ 5·4). The concentration of low-sulphur protein in the precipitate was obtained from the difference between these two readings ($E^{1\%}_{1\,cm.}$ 8·6). Untreated wool: ○, low-sulphur fraction; △, high-sulphur fraction. Reduced and alkylated wool: ●, low-sulphur fraction; ▲, high-sulphur fraction.

Table 1. *Comparison of protein yields and of sulphur contents in fractions obtained from reduced and alkylated wool and from untreated wool*

Fractionation procedures are described under Methods.

Comparable prep. no. in Tables 2 and 3	Fraction component		Yield of protein (%)		Sulphur content (%)	
			Untreated wool	Reduced and alkylated wool	Untreated wool	Reduced and alkylated wool
2, 3, 6	L1	Low-S	63	58	2·7	2·6
		High-S			5·4	5·6
	L1R	Residue	—	—	3·5	3·3
7	L1	Low-S	63	57	2·7	2·6
		High-S			5·3	5·4
	L2	Low-S	15	12	2·6	2·7
		High-S			5·5	5·6
	L2R	Residue	—	—	3·5	3·3
4	M1	Low-S	53	47	2·7	2·5
		High-S			5·3	5·4
	M2	Low-S	9	10	2·3	2·2
		High-S			5·5	5·3
	M3	Low-S	11	9	2·6	2·6
		High-S			5·5	5·6
	M4	Low-+high-S	—	—	3·5	3·4
	M3R	Residue				

Table 2. *Specific radioactivities of* S*-carboxymethylcysteine in fractions obtained from wool reduced at* pH 5·0 *for* 18 *hr. at* 20° *with* M*-sodium mercaptoacetate*

Fractionation procedures and *S*-carboxymethylcysteine (SCMC) isolation and determinations are described under Methods. Reductions were performed with the following amounts of wool: 1 g. (prep. 2 and 3), 10 g. (prep. 4) and 5 g. (prep. 5.) Addition of carrier: 200 μmoles/μc of ^{14}C (prep. 2) and 100 μmoles/μc of ^{14}C (prep. 3, 4 and 5). Iodoacetate uptake by the wool: 600 μmoles (prep. 2), 580 μmoles (prep. 3), 5·8 m-moles (prep. 4) and 3·05 m-moles (prep. 5).

Prep. no.	Fraction	Component	Specific radioactivity (μc/m-mole of SCMC)	Cystine residues reduced (%)
2	L1	Low-S	2·2	44
		High-S	2·4	48
	L1R	Residue	3·1	62
3	L1	Low-S	4·7	47
		High-S	4·7	47
	L1R	Residue	6·1	61
4	M1	Low-S	4·7	47
		High-S	4·7	47
	M2	Low-S	4·5	45
		High-S	4·6	46
	M3	Low-S	3·8	38
		High-S	3·7	37
	M4	Low-+high-S	3·7	37
	M3R	Residue	4·2	42
5	M1*	Low-+high-S	5·6	56
	M1R	Residue	6·6	66

* This fraction comprises the first pH 10·5 (initial pH) extract and contains 25% of the total wool protein.

Table 3. *Specific radioactivities of* S*-carboxymethylcysteine in fractions obtained from wool reduced at* pH 5·0 *for* 2 *hr. at* 10° *with* M*-sodium mercaptoacetate*

Fractionation procedures and *S*-carboxymethylcysteine (SCMC) isolation and determination are described under Methods. Reductions were performed with the following amounts of wool: 1 g. (prep. 6) and 5 g. (prep. 7). Addition of carrier: 50 and 25 μmoles/μc of ^{14}C respectively. Iodoacetate uptake by the wool: 140 and 300 μmoles respectively.

Prep. no.	Fraction	Component	Specific radioactivity (μc/m-mole of SCMC)	Cystine residues reduced (%)
6	L1	Low-S	0·9	4·5
		High-S	0·8	4·0
	L1R	Residue	0·9	4·5
7	L1	Low-S	1·1	2·5
		High-S	1·0	2·5
	L2	Low-S	1·3	3·0
		High-S	1·2	3·0
	L2R	Residue	2·0	5·0

Table 4. *Attempts at complete reduction of wool at* pH 5·0

Disulphide fission by bisulphite and mercaptoacetate was carried out as described under Methods.

Reagent	Concn. (M)	No. of treatments	Residual disulphide (μmoles/g.)	Reduction† (%)
Sodium bisulphite + Neohydrin*	0·20 + 0·01	1	74	85
Mercaptoacetate	1·00	4	57	89

* 3-Chloromercuri-2-methoxypropylurea.

† It is assumed that no mixed disulphide is present, so that the figures given represent minimal values.

are of the same order of magnitude. In the most extensively fractionated preparation 4, the spread of activities varies by only about ± 10 % from the mean. These observations also apply to proteins extracted from wools reduced to only about 6 % (Table 3). In examining the differences between extracts, no consistent pattern can be discerned; for example, in preparations 2 and 3 the residues have higher specific activities than the soluble proteins, but this is not true in preparation 4. Similarly, in the last-named preparation the earliest fractions were more highly labelled than the later ones, whereas in preparation 5 the reverse was true.

These experiments were done on a small scale and it was difficult to recover quantitatively the extracted and fractionated proteins. Nevertheless, the yields actually obtained were similar in magnitude to those determined separately on a larger scale with identically treated non-radioactive wool (Table 1).

Attempts to obtain complete fission of disulphide bonds at pH 5·0. The results in Table 4 show that it is possible to break more than 50 % of the disulphide bonds in wool by multiple reduction with mercaptoacetate under nitrogen or by reaction with sulphite in the presence of an organic mercurial.

DISCUSSION

The results presented in Tables 2 and 3 show that there is no difference between the specific activities of the *S*-carboxymethylcysteine derived from high- and low-sulphur components of a single fraction and that only small variations occur in the radioactivity of successive extracts. Thus all the protein disulphide in wool is fairly uniformly reduced at pH 5·0.

In considering the morphological location of the easily reducible disulphide, it was important to establish that the extraction of wool protein from the modified fibre followed a normal pattern. The results in Table 1 and Fig. 1 indicate that this assumption can be made.

It has been reported that the most readily extractable protein originates within the *ortho*-cortex of the fibre (Fraser & Rogers, 1953; Mercer, 1954). If this is so, then the initial alkaline mercaptoacetate extraction (Fraction M1, preparations 4 and 5 in Table 2) would preferentially isolate protein from the *ortho*-region and the last (Fraction M3, preparation 4) from the *para*-region. Since the *S*-carboxymethylcysteines from these extracts have similar specific activities, it then follows that the reactive disulphide is not preferentially located in the *ortho*-region of the

wool fibre as was suggested by Mercer, Golden & Jeffries (1954) and Alexander & Fox (1956).

It is generally accepted that the low-sulphur proteins originate in the microfibrils and the high-sulphur proteins from within the matrix (Birbeck & Mercer, 1957; Rogers, 1959). If this is true, the reactive disulphide is not preferentially located in either the microfibrils or the matrix, because the high- and low-sulphur-containing components of each extract were equally labelled. This would clarify the situation created by the conflicting suggestions of Earland & Wiseman (1959) and Blackburn (1960) that the (A+B) disulphide fraction preferentially resides in the microfibrils and the counter-proposal of Jeffrey, Sikorski & Woods (1956), Lindley (1959), Rogers (1959), and Fraser, MacRae & Rogers (1959, 1960) that these disulphides are concentrated in the matrix.

From our present knowledge of the localization of proteins within the wool fibre it would appear that morphological factors do not influence the reactivity of wool cystine residues. Wool can thus be regarded as behaving similarly to other proteins, which are also incompletely reduced by sulphite or mercaptoacetate under certain conditions (Lindley, 1948; Cecil & McPhee, 1959; Cecil & Loening, 1960).

In interpreting the results of experiments on the fission of disulphide bonds in wool, Phillips (1946) and Lindley (1959) had suggested that there was a sharp division into reactive and non-reactive fractions. It now seems unlikely that this 50% reduction stage is an absolute division for, as many workers have found, the removal of one of the reaction products or an increase in the amount of reductant results in an increase in the extent of reduction. The evidence for this is as follows. Wool successively treated at pH 5·6 with mercaptoacetate, followed by alkylation after each reduction, is completely reduced (O'Donnell, 1954); successive reductions with a large excess of mercaptoacetate in the absence of air gives an 88% reduction of the disulphide bonds at pH 6·5 (Leach & O'Donnell, 1961) and at pH 5·0 (Table 4). Similar evidence comes from the almost complete fission of disulphide linkages in wool by sulphite in the presence of mercurial compounds at pH 5·0 (Table 4) or at pH 7·0 (Leach, 1959, 1960), by cuprammonium sulphite at pH 8·0 (Swan, 1957) and by sulphite–tetrathionate reagent at pH 5·0 (J. M. Swan, personal communication). By these means the so-called (C+D) fraction of wool disulphide, formerly considered completely non-reactive, can be fully broken. The above evidence suggests that all the disulphide groups of wool are accessible to these reagents and that disulphide fission by them is normally limited by an equilibrium reaction. This may also apply to other proteins; for example, the finding by Leach (1960) that disulphide bonds in serum albumin can also be ruptured at pH 7·0 by bisulphite solutions containing mercurials favours the equilibrium concept rather than the idea of steric inaccessibility proposed by Alexander & Hamilton (1960) for this protein.

If all the cystine residues were equally reactive, complete reduction should be somewhat easier to achieve than it is (Table 4). This suggests that the unreduced disulphides have lower oxidation–reduction potentials than the reactive disulphides. One might expect a gradation of redox potentials in disulphide bonds depending on the amino acid environment and the tertiary structure of the protein.

Variations in structure have relatively little effect on the oxidation–reduction potentials in simple thiol–disulphide systems and with small cystine-containing peptides, values around +0·1 (versus the normal hydrogen electrode) being invariably obtained (Cecil & McPhee, 1959; Eldjarn & Pihl, 1957; W. E. Savige, personal communication). Yet the introduction of a cyclic structure, as in α-lipoic acid, yields a system with an oxidation–reduction potential of −0·42 v (Reed, 1957). By analogy one might expect to observe some differences between inter- and intra-chain-linked disulphide bonds. Cecil & Loening (1960) have indeed shown that substantial differences in the reducibility of the two types of disulphide bonds do exist in insulin. Although Lindley (1956) has suggested that disulphide reactivity differences in wool might be due to the presence of inter- and intra-chain-bonded cystine residues, the extent to which these might be present in wool is still a matter of controversy (Earland & Wiseman, 1959; Crewther & Dowling, 1960).

Another important factor determining the reactivity of disulphide groups is the manner in which the polypeptide chains are folded, as demonstrated by the ease of reducibility in the presence of urea or guanidine (Markus & Karush, 1957; Katchalski, Benjamin & Gross, 1957). In addition Farnworth (1955) has shown that induced variations in the environment of the cystine affect the apparent amount of the (A+B) fraction.

The experiments performed at different levels of reduction (Tables 2 and 3) indicate that the disulphides in the two major wool proteins are not only equally reduced at equilibrium, but that the rates of fission, over the range studied, are probably also equal. These two proteins differ markedly in amino acid composition, in net charge at pH 5·0 (Corfield, Robson & Skinner, 1958; Gillespie & Simmonds, 1960) and possibly also in tertiary structure (Crewther & Dowling, 1960). It is surprising therefore that the cystine residues in

these two proteins have similar reactivities. It may be that this equivalence is fortuitous, resulting from a number of compensating factors; alternatively the tertiary structure of the two proteins is similar or else the cystine residues in these two proteins may share a common environment. The latter two possibilities could be accommodated if both types of wool protein either had their cystine residues in close contact (R. D. B. Fraser, personal communication) or if each protein was present in both the microfibrils and the matrix. At present it is not possible to decide whether any one of these suggestions provides the correct explanation.

SUMMARY

1. Wool was reduced between 6 and 60% at pH 5·0 with mercaptoacetate, alkylated with [2-^{14}C]iodoacetate and fractionally extracted with alkaline mercaptoacetate. After alkylation with unlabelled iodoacetate these fractions were separated by acid precipitation into proteins of high- and low-sulphur content. The extent of reduction at pH 5·0 in each constituent was determined by isolating the *S*-carboxymethylcysteine and measuring its specific radioactivity.

2. The disulphide bonds in the proteins of high- and low-sulphur content from any particular fraction were reduced to the same extent. Variations between the fractions themselves were quite small, usually less than 5% and not deviating by more than 10% from the mean.

3. It was concluded that the cystine residues of the so-called (A+B) fraction are not confined to any particular protein component and it is inferred that they are equally distributed throughout the morphological components of wool. The apparent differences in reactivity between disulphide bonds in wool have been interpreted as resulting from an equilibrium between the SS–SH system and the reducing agents used.

We wish to thank Dr H. Lindley and Dr S. J. Leach for extremely helpful discussions and the latter also for disulphide determinations. Thanks are also due to Dr K. W. Zimmermann for sulphur analyses and Mr L. H. T. West for technical assistance.

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APPENDIX

The Equilibrium between the Disulphide Bonds in Wool and Mercaptoacetate

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One of us (O'Donnell, 1954) has shown that it is possible to approach complete reduction of the disulphide bonds in wool by successive treatments, each involving reduction with mercaptoacetate at pH 5·6 followed by alkylation of the SH groups produced. Since this was apparently effective because alkylation at each step removed one of the products, namely the SH groups in the wool, it seemed likely that the reduction reaction was in fact an equilibrium between two redox systems. In this event complete reduction of the disulphide bonds should be attainable either by removing the other product of reaction, namely biscarboxymethyl disulphide, or by greatly increasing the ratio of reducing agent to wool.

Wool (0·5 g., Merino 64's) was treated in an atmosphere of N_2 at 28° for at least 6 hr. with various amounts of mercaptoacetic acid buffered at pH 6·8–6·9 with 0·05 M-phosphate. The wool was then alkylated with iodoacetate at pH 8·0–8·5 and analysed for S·S groups by the method of Leach (1959, 1960).

Table 1 shows that the extent of reduction of the disulphide bonds in wool can be made to approach completion either by increasing the concentration of reducing agent (Expts. 1 and 2) or by increasing the total amount of mercaptoacetate and replenishing it twice (Expt. 3) or four times (Expt. 4) to remove oxidation products. With the consecutive treatments of the wool only the last was followed by alkylation with iodoacetate. The results suggest that the extent of reduction is normally limited by an equilibrium between the S·S bonds and mercaptoacetate.

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Table 1. *Extent of reduction of wool by mercaptoacetate*

Expt. no.	Concn. of mercaptoacetate (M)	Ratio of soln. to wool (ml./g.)	Disulphide remaining (μmoles/g. of wool)	Reduction* (%)
—	0	—	500	0
1	0·10	100:1	310	38
2	0·40	100:1	182	64
3	0·40	2000:1 (twice)	82	82
4	0·40	200:1 (four times)	55	88

* These figures are minimal since, in calculating the percentage reduction, it is assumed that no mixed disulphides between the wool and mercaptoacetic acid are left on the wool. With large excesses of reducing agent these products are less likely to be of importance.

Biochem. J. (1961) **79**, 288

Studies on Glucosaminidase

5. KIDNEY *N*-ACETYL-β-GLUCOSAMINIDASE AND *N*-ACETYL-β-GALACTOSAMINIDASE*

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The enzyme *N*-acetyl-β-D-galactosaminidase has been studied in a rat-kidney preparation which was known to have *N*-acetyl-β-D-glucosaminidase activity (Pugh, Leaback & Walker, 1957). It was not found possible to separate the two activities by various fractionation procedures. The two *p*-nitrophenyl hexosaminides which were used as substrates competed but not in the simple manner found with a testicular extract (Woollen, Heyworth & Walker, 1961).

A preliminary account of part of this work has appeared (Woollen, Heyworth & Walker, 1959).

MATERIALS AND METHODS

Enzyme preparation. Fresh rat kidney was weighed and ground for 1 min. with water in a glass homogenizer and brought to pH 4·5 with 0·05M-sodium citrate–citric acid buffer and a final concentration of 10% (w/v). The mixture was centrifuged (1500*g*) and the supernatant allowed to stand for 1 day at 4° before use. Dilutions were made from this stock solution in 0·05M-citrate buffer, pH 4·5, when required, immediately before use.

Attempts to use enzyme preparations which had been partially purified by ammonium sulphate fractionation were made difficult by the occasional inconsistency of duplicate determinations of enzymic activities.

Substrates, inhibitors and assay procedure. These are described by Woollen *et al.* (1961). Except where otherwise indicated 0·1 ml. of enzyme solution was incubated with substrate in 0·05M-citrate buffer, pH 4·52, for 30 min. at 37° in a total volume of 1·0 ml. These conditions were chosen after initial experiments described under Results.

In experiments where mixtures of phenyl and *p*-nitrophenyl substrates were used it was possible to estimate the liberated phenol and *p*-nitrophenol separately since *p*-nitrophenol gives no colour under the conditions of phenol estimation (Folin & Denis, 1912) and phenol gives no colour under the conditions of *p*-nitrophenol estimation.

RESULTS

Effect of pH, *enzyme concentration and time of incubation*

Fig. 1 shows the effect of pH on *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities. The general shape of the pH curves was similar for the two activities.

Both enzyme activities were proportional to the enzyme present over a fourfold range of enzyme concentration with the substrates *p*-nitrophenyl *N*-acetylglucosaminide (3·6 mM) and *p*-nitrophenyl *N*-acetylgalactosaminide (2·2 mM). With the same substrates both activities were proportional to time of incubation over the period tested (1 hr.).

Attempts to separate the two activities

Fractionation and partial inactivation of the crude kidney-enzyme preparation were carried out in the same way as with a testicular-enzyme preparation (Woollen *et al.* 1961). The ratios of *N*-

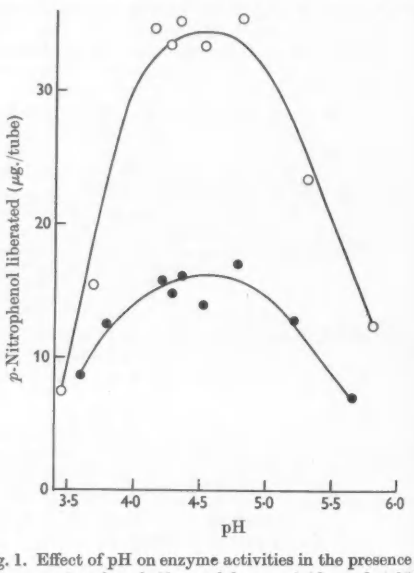

Fig. 1. Effect of pH on enzyme activities in the presence of 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 0·25% (w/v) of enzyme (○) or 2·2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide and 1·25% (w/v) of enzyme (●). For other conditions see text.

* Part 4: Leaback & Walker (1961).

Table 1. *Effect of ammonium sulphate fractionation on* N-*acetylglucosaminidase and* N-*acetylgalactosaminidase activities*

Activities were measured with either 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Enzyme preparation	*N*-Acetylglucosaminidase (% of activity of untreated preparation)	Ratio *N*-acetylglucosaminidase: *N*-acetylgalactosaminidase activities
Untreated	100·0*	8·2
$(NH_4)_2SO_4$ fractions:†		
0–30 %	6·7	8·8
30–40 %	3·3	8·0
40–50 %	19·3	8·3
50–60 %	8·0	7·7

* 66·6 μg. of *p*-nitrophenol liberated/tube.
† Precipitated between the indicated limits of percentage saturation with $(NH_4)_2SO_4$.

Table 2. *Adsorption of* N-*acetylglucosaminidase and* N-*acetylgalactosaminidase activities by calcium phosphate gel*

Residual activities in the supernatants were measured with either 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide as substrate. For other conditions see text.

Total gel added (ml./10 ml. of enzyme solution)	*N*-Acetylglucosaminidase (% of initial activity)	Ratio *N*-acetylglucosaminidase: *N*-acetylgalactosaminidase activities
0	100*	8·5
0·5	91	8·8
0·6	44	9·0
0·7	34	9·3
0·8	17	8·7
0·9	6	9·0

* 32·9 μg. of *p*-nitrophenol liberated/tube.

Table 3. *Effect of partial heat inactivation on* N-*acetylglucosaminidase and* N-*acetylgalactosaminidase activities*

The enzyme preparation was preincubated in 0·05 M-citrate buffer, pH 4·5, at 56° for various times in the absence of substrate. Activities were then measured with 2·2 mM-*p*-nitrophenyl *N*-acetylglucosaminide or *N*-acetylgalactosaminide in the usual way.

Period of preincubation (min.)	*N*-Acetylglucosaminidase (% of initial activity)	Ratio *N*-acetylglucosaminidase: *N*-acetylgalactosaminidase activities
0	100*	7·9
15	52	8·1
30	29	7·0
60	15	6·9

* 78·6 μg. of *p*-nitrophenol liberated/tube.

acetylglucosaminidase:*N*-acetylgalactosaminidase, measured under standard conditions, were constant both on ammonium sulphate fractionation (Table 1) and with progressive adsorption (Table 2) on calcium phosphate gel (Keilin & Hartree, 1951). On partial heat inactivation to 15 % of the initial activity the ratio again remained constant (Table 3).

Kinetic experiments

The above results indicated that a single enzyme protein in rat kidney had both *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities. Kinetic experiments were then undertaken to study the nature of the combination of the enzyme with the respective substrates.

Effect of substrate concentration. K_m and $V_{max.}$ values (Table 4) were determined according to Lineweaver & Burk (1934) from typical straight-line plots for each of the four substrates.

Competitive behaviour. With the *p*-nitrophenyl substrates, both activities were competitively inhibited by *N*-acetylglucosamine, 2-acetamido-2-deoxygluconolactone and phenyl *N*-acetyl-β-D-glucosaminide and by the corresponding galactosamine derivatives (cf. Woollen *et al.* 1961). The K_i values (Table 5), however, show marked differences when measured against the two respective substrates. By comparing Tables 4 and 5 it can be seen that the K_i values for the two phenyl *N*-acetylhexosaminides agree more closely with the K_m values when K_i is measured in the presence of the analogous *p*-nitrophenyl *N*-acetylhexosaminide as substrate.

Competing p-*nitrophenyl substrates.* Fig. 2 shows Lineweaver & Burk plots for the two separate *p*-nitrophenyl *N*-acetylhexosaminides. From the K_m and $V_{max.}$ values derived from these plots the theoretical line was calculated for equimolar mixtures on the assumption of pure competition between these two substrates (Thorn, 1949; Foster & Niemann, 1951). The experimental plot for equimolar mixtures does not agree with this theoretical plot. On the other hand, the total hydrolysis in the mixture is non-additive, so the hydrolysis of the individual substrates is not independent.

Variation of inhibitor concentration. Fig. 3 shows the effect of variation of the concentration of three substances having an inhibitory effect on *N*-acetylglucosaminidase. There is no suggestion of departure from linearity in these plots and therefore no indication that the inhibition is of a partially competitive type (Dixon & Webb, 1958).

Competing phenyl and p-*nitrophenyl substrates.* Fig. 4 shows the results of experiments from which K_m values were determined for *p*-nitrophenyl *N*-acetylgalactosaminide and phenyl *N*-acetylglucosaminide. At the same time K_i values for each were

Table 4. *K_m and $V_{max.}$ values*

Values of K_m and $V_{max.}$ were determined by the method of Lineweaver & Burk (1934). For conditions of assay see text. $V_{max.}$ values are expressed as μmoles of substrate hydrolysed/mg. of fresh kidney extracted. Values are given as means ± S.E. with no. of results in parentheses.

Substrate	Concentration range (mM)	K_m (mM)	$V_{max.}$ (μmole/mg.)
p-Nitrophenyl *N*-acetylglucosaminide	0·73–5·84	0·72 ± 0·09 (17)	0·156 ± 0·012 (17)
p-Nitrophenyl *N*-acetylgalactosaminide	0·36–2·56	0·40 ± 0·09 (17)	0·016 ± 0·001 (17)
Phenyl *N*-acetylglucosaminide	1·00–16·00	1·32 ± 0·13 (7)	0·087 ± 0·012 (7)
Phenyl *N*-acetylgalactosaminide	1·00–16·00	1·02 ± 0·22 (4)	0·017 ± 0·003 (4)

Table 5. *K_i values for various competing substances*

K_i values were evaluated by the method of Lineweaver & Burk (1934). The substrates were (*a*) *p*-nitrophenyl *N*-acetylglucosaminide (0·73–5·84 mM) or (*b*) *p*-nitrophenyl *N*-acetylgalactosaminide (0·18–1·46 mM). For other conditions see text.

	K_i (mM)	
Inhibitors	(*a*)	(*b*)
N-Acetylglucosamine	3·1	7·6
N-Acetylgalactosamine	0·4	1·6
2-Acetamido-2-deoxygluconolactone	0·0007	0·0049
2-Acetamido-2-deoxygalactonolactone	0·0005	0·0018
Competing substrates		
Phenyl *N*-acetyl-β-D-glucosaminide	0·9	2·6
Phenyl *N*-acetyl-β-D-galactosaminide	0·2	0·6

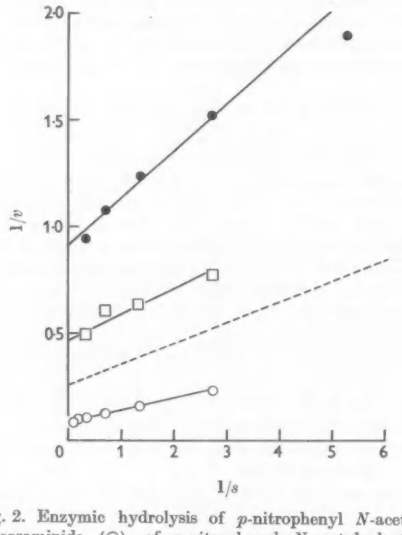

Fig. 2. Enzymic hydrolysis of *p*-nitrophenyl *N*-acetylglucosaminide (○), of *p*-nitrophenyl *N*-acetylgalactosaminide (●) and of equimolar mixtures of the two (□). *v*, Total *p*-nitrophenol liberated (μg./tube); *s*, total substrate concn. (mM); ---, theoretical plot for equimolar mixtures on the assumption of pure competition at a single enzyme site. For conditions see text.

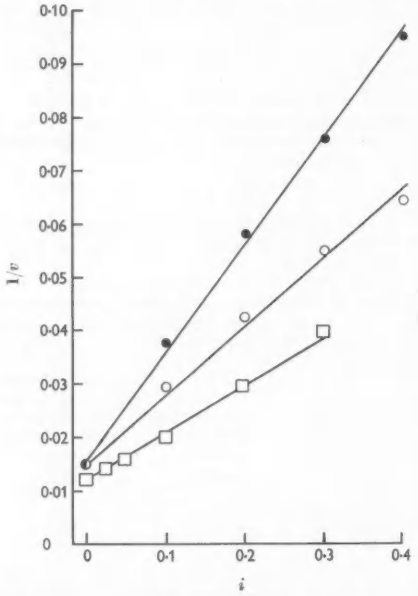

Fig. 3. Effect of inhibitor concentration on enzymic hydrolysis. Substrate: *p*-nitrophenyl *N*-acetylglucosaminide (1·45 mM); inhibitors: ○, *N*-acetylglucosamine (0·15 M); ●, *N*-acetylgalactosamine (0·045 M). Substrate: phenyl *N*-acetylglucosaminide (8 mM); inhibitor: □, *p*-nitrophenyl *N*-acetylgalactosaminide (0·004 M). *v*, *p*-Nitrophenol or phenol liberated (μg./tube); *i*, inhibitor solution in assay mixture (ml.). For other conditions see text.

determined against the other substrate, by the method of Lineweaver & Burk (1934). From these values and the $V_{max.}$ values the theoretical lines were calculated from equations (1) and (2) (Theory section). There is reasonable agreement of these lines with the experimental results obtained with mixtures of these substrates.

Table 6 gives the values of K_m for phenyl *N*-acetylglucosaminide (A) and *p*-nitrophenyl *N*-acetylgalactosaminide (B) and of K_i for these sub-

Table 6. *K_m and K_i values and their ratios*

Phenyl *N*-acetylglucosaminide (A) was the substrate in Expt. 1 over the range 1–16 mM and the inhibitor in Expt. 2 at 4, 8 and 16 mM. *p*-Nitrophenyl *N*-acetylgalactosaminide (B) was the substrate in Expt. 2 over the range 0·18–2·92 mM and the inhibitor in Expt. 1 at 0·04, 0·07 and 0·29 mM. The three K_i values were averaged in each case.

	Expt. 1			Expt. 2		
	K_m (mM)	K_i (mM)	K_{mA}/K_{iB}	K_m (mM)	K_i (mM)	K_{iA}/K_{mB}
Phenyl *N*-acetylglucosaminide (A)	1·1	—	15·7	—	3·5	15·9
p-Nitrophenyl *N*-acetylgalactosaminide (B)	—	0·07		0·22	—	

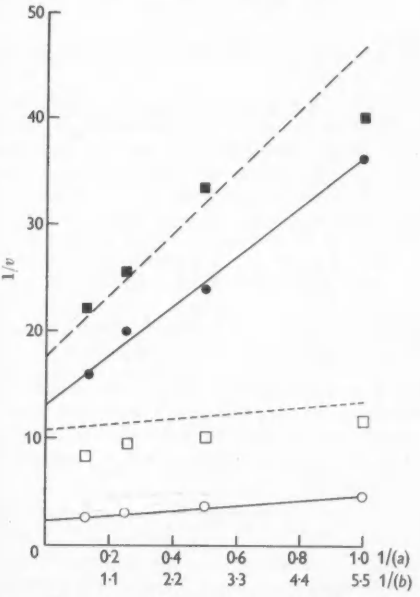

Fig. 4. Enzymic hydrolysis of phenyl *N*-acetylglucosaminide (○), of *p*-nitrophenyl *N*-acetylgalactosaminide (●) and of mixtures of the two. *v*, *p*-Nitrophenol or phenol liberated (μmoles/tube); (*a*) concn. (mM) of phenyl *N*-acetylglucosaminide; (*b*) concn. (mM) of *p*-nitrophenyl *N*-acetylgalactosaminide. □, Liberation of phenol and ■, liberation of *p*-nitrophenol from mixtures containing a ratio (*a*:*b*) of 5·5:1. Liberation of phenol (---) and of *p*-nitrophenol (———) from these mixtures calculated according to the text (equations (1) and (2)).

stances when used as inhibitors of the other. The K_i values do not agree with the respective K_m values, but the ratios K_{mA}/K_{iB} and K_{iA}/K_{mB} are equal (see below).

THEORY

The effects on the reaction velocity of varying substrate concentration in the absence and in the presence of a fixed concentration of inhibitor and of varying inhibitor concentration at fixed substrate concentration resemble formally those given by an enzyme with a single active site and a purely competitive type of inhibitor, as found with a testicular preparation (Woollen *et al.* 1961). With the kidney preparation, however, different K_i values for competitive inhibitors were obtained according to the substrate employed (Table 5). Also the K_m value for *N*-acetylglucosaminide differed from the K_i value when K_i was measured against *N*-acetylgalactosaminide as substrate, and vice versa (Tables 4 and 5). These results cannot be explained on the basis of pure competition at a single site. Two alternative reaction schemes, each of which is compatible with the results, are analysed below. The first permits the conventional formulation of the reactions between enzyme and substrate or inhibitor to be retained but requires that the enzyme has two active sites with special and restricted properties. The second requires only a single active site but includes an additional displacement reaction which permits competition between substrate and inhibitor after, as well as before, combination with the enzyme. It is shown that with competing substrates such a reaction would, in certain circumstances, give rise to the simple quantitative relationship between K_m and K_i values that was observed.

Scheme 1; two specific sites with cross-inhibition. The enzyme has one site at which only *N*-acetylglucosaminides and another at which only *N*-acetylgalactosaminides are broken down. The two sites are completely independent but in a mixture of substrates each substrate acts as a purely competitive inhibitor of the hydrolysis of the other.

Let A and B be the two substrates with concentrations a and b respectively. Let K_{1A} and K_{1B} be the respective Michaelis and inhibitor constants at site 1 and K_{2B} and K_{2A} the respective Michaelis and inhibitor constants at site 2. Let v_A, v_B, V_A, V_B be the initial and the maximum velocities for the enzymic hydrolysis of the two substrates. Then

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{a}\frac{K_{1A}}{V_A}\left(1 + \frac{b}{K_{1B}}\right)$$

and $$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{b}\frac{K_{2B}}{V_B}\left(1 + \frac{a}{K_{2A}}\right).$$

Provided that the hydrolysis of each substrate can be estimated in the presence of the other the values K_{1B} and K_{2A} may be determined by the method of Lineweaver & Burk (1934). If then the rates of hydrolysis of the individual substrates are studied in mixtures of the two, such that $b = xa$, the results may be compared with the theoretically expected rates which are given by equations (1) and (2).

$$\frac{1}{v_A} = \frac{1}{V_A}\left(1 + x\frac{K_{1A}}{K_{1B}}\right) + \frac{1}{a}\frac{K_{1A}}{V_A}, \quad (1)$$

and $$\frac{1}{v_B} = \frac{1}{V_B}\left(1 + \frac{1}{x}\frac{K_{2B}}{K_{2A}}\right) + \frac{1}{b}\frac{K_{2B}}{V_B}. \quad (2)$$

Scheme 2; single site with displacement. Each substrate combines with the enzyme E at the same active site. When present together the substrates compete for this site and may also displace each other from it after combination with the enzyme. The reactions, with velocity constants and with concentrations given below, may be formulated thus:

$$\underset{e}{E} + \underset{a}{A} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \underset{p}{EA} \xrightarrow{k_2} E + \text{products} \quad (3)$$

$$\underset{e}{E} + \underset{b}{B} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \underset{q}{EB} \xrightarrow{k_4} E + \text{products} \quad (4)$$

$$\underset{q}{EB} + \underset{a}{A} \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} \underset{p}{EA} + \underset{b}{B} \quad (5)$$

If v_A and v_B are the initial velocities of breakdown of EA and EB, V_A and V_B the maximum velocities and the total enzyme

$$e_T = e + p + q \quad (6)$$

then $$v_A = k_2 p \quad (7)$$

$$v_B = k_4 q \quad (8)$$

$$V_A = k_2 e_T \quad (9)$$

$$V_B = k_4 e_T. \quad (10)$$

The respective Michaelis constants, K_{mA} and K_{mB}, may be measured in the presence of each substrate alone, when

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{aV_A}\frac{k_2 + k_{-1}}{k_1} \quad (11)$$

$$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{bV_B}\frac{k_4 + k_{-3}}{k_3} \quad (12)$$

$$K_{mA} = \frac{k_2 + k_{-1}}{k_1} \quad (13)$$

$$K_{mB} = \frac{k_4 + k_{-3}}{k_3}. \quad (14)$$

The solutions of the steady-state equations when both substrates are present is complex. A detailed analysis for a substrate (S) and an inhibitor (I) (Reiner, 1959) shows that the displacement reaction ($ES + I \rightleftarrows EI + S$) leads in general to a picture which differs from a purely competitive type of inhibition.

However, in the special case of the above scheme which is relevant to the present discussion, i.e. when $k_5 = k_1$ and $k_{-5} = k_3$, then (5) becomes

$$\underset{q}{EB} + \underset{a}{A} \underset{k_3}{\overset{k_1}{\rightleftarrows}} \underset{p}{EA} + \underset{b}{B} \quad (15)$$

and, in the steady state, from reactions (3), (4) and (15)

$$\frac{dp}{dt} = 0 = k_1 a(e+q) - p(k_2 + k_{-1} + k_3 b)$$

and $$\frac{dq}{dt} = 0 = k_3 b(e+p) - q(k_4 + k_{-3} + k_1 a);$$

from which, with equations (6)–(10),

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{1}{a}\frac{1}{V_A}\left(\frac{k_2 + k_{-1} + k_3 b}{k_1}\right) \quad (16)$$

and $$\frac{1}{v_B} = \frac{1}{V_B} + \frac{1}{b}\frac{1}{V_B}\left(\frac{k_4 + k_{-3} + k_1 a}{k_3}\right) \quad (17)$$

and in an experiment in which a is varied in the absence and in the presence of a fixed concentration b, equations (11) and (16) show that plots of $1/v_A$ versus $1/a$ give the appearance of competitive inhibition with

$$'K_{iB}' = \frac{k_2 + k_{-1}}{k_3}. \quad (18)$$

Similarly, with a fixed concentration a and varying concentration b, equations (12) and (17) would give

$$'K_{iA}' = \frac{k_4 + k_{-3}}{k_1}. \quad (19)$$

From equations (13), (14), (18) and (19)

$$\frac{K_{mA}}{'K_{iB}'} = \frac{'K_{iA}'}{K_{mB}} = \frac{k_3}{k_1} = \text{a constant}. \quad (20)$$

Such a constant was found experimentally (Table 6). Equations (16) and (17) may be written

$$\frac{1}{v_A} = \frac{1}{V_A}\left(1 + \frac{K_{mA}}{a}\right) + b\frac{k_3}{k_1}\frac{1}{aV_A}$$

and $$\frac{1}{v_B} = \frac{1}{V_B}\left(1 + \frac{K_{mB}}{b}\right) + a\frac{k_1}{k_3}\frac{1}{bV_B}$$

showing that plots of $1/v_A$ versus b and of $1/v_B$ versus a are straight lines and the inhibition is therefore of the purely competitive type (cf. Fig. 3).

Now, consider mixed substrate experiments in which $b = xa$. From (13) and (16)

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{k_3 b}{k_1 a V_A}$$

Substituting, $b = xa$,

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{k_3 x}{k_1 V_A}$$

Substituting for k_3/k_1, from (20),

$$\frac{1}{v_A} = \frac{1}{V_A} + \frac{K_{mA}}{aV_A} + \frac{xK_{mA}}{`K_{iB}' V_A}$$

or

$$\frac{1}{v_A} = \frac{1}{V_A}\left(1 + \frac{xK_{mA}}{`K_{iB}'}\right) + \frac{1}{a}\frac{K_{mA}}{V_A}. \qquad (21)$$

This is the same as (1), so that with mixed-substrate experiments the predicted velocities of breakdown of the individual substrates are the same as those derived from scheme 1.

If, in the scheme described in reactions (3)–(5), the relationship $K_{mA} = K_{mB} \cdot K_D$ holds (where $K_D = k_{-5}/k_5$), either by coincidence or by virtue of $k_2 \ll k_{-1}$ and $k_4 \ll k_{-3}$, the effect of one substrate on the rate of hydrolysis of the other is not affected by the occurrence of the displacement reaction. Such a system cannot be differentiated from the case of simple competition without a displacement reaction.

DISCUSSION

A crude enzyme preparation was used for this work, but experience with partially purified ram-testicular enzyme (Woollen *et al.* 1961) suggests that the difficulties encountered with a purified preparation may well, in future, be overcome by the presence of dilute albumin or Triton X-100 when diluting the purified enzyme.

Attempts to separate the two enzyme activities *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase were as unsuccessful with rat-kidney extract as were those with a preparation from ram testis, and it is concluded that both activities are associated with the same enzyme protein.

In experiments with the two *p*-nitrophenyl substrates both activities were competitively inhibited by *N*-acetylglucosamine, 2-acetamido-2-deoxygluconolactone and phenyl *N*-acetyl-β-D-glucosaminide and by the corresponding galactosamine derivatives, but the K_i values for a given inhibitor against each substrate were not equal (Table 5). The experiment with competing substrates confirmed that the mechanism of the reaction was not one of pure competition at a single site (Fig. 2).

Experiments with inhibitors at various concentrations (Fig. 3) gave no indication that the competition was of a partial rather than a pure type (Dixon & Webb, 1958).

Two theoretical reaction schemes which meet the requirements of these results were analysed. Both led to identical predicted velocities of breakdown in mixtures of two substrates which were in reasonable agreement with the experimental results (Fig. 4). However, the second scheme, which postulated a single active site at which a displacement mechanism operated in addition to the usual competition, also required a relationship between the Michaelis and inhibitor constants for competing substrates. This relationship was observed experimentally.

The restriction on the values of the rate constants which was introduced in deriving the above scheme requires the rate of formation of enzyme–substrate complex to be the same whether the substrate reacts with the free enzyme or displaces another substrate from its complex with the enzyme. This requirement may be less improbable than appears at first sight. The reactions may be considerably more complex than those formulated, which involve only single intermediate enzyme–substrate complexes, and it is possible that the reaction between free enzyme and substrate and the displacement reaction share a common rate-limiting step.

For the particular scheme of displacement suggested here it is necessary to measure Michaelis and inhibitor constants of two competing substrates. Reiner (1959) shows the circumstances in which a general inhibitor mechanism involving displacement gives different experimental results from other inhibitor mechanisms. It may well be that these mechanisms involving displacement may be more commonly found by suitable analysis of enzyme systems where Michaelis and inhibitor constants of substrates have been at variance.

SUMMARY

1. Two *N*-acetylhexosaminidase activities of extracts of rat kidney have been measured with the substrates *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide.

2. No separation of the activities was achieved by fractionation with ammonium sulphate or with calcium phosphate gel or by partial heat inactivation.

3. The K_i values of six competing substances, *N*-acetylglucosamine, 2-acetamido-2-deoxygluconolactone, phenyl *N*-acetylglucosaminide and the corresponding galactosamine derivatives were not the same when measured against *p*-nitrophenyl *N*-acetylglucosaminide and *N*-acetylgalactosaminide respectively.

4. There was no evidence of partially competitive inhibition.

5. Two schemes are suggested for the enzyme mechanism. The first is of two independent sites for the two activities on the same enzyme protein, together with inhibition of *N*-acetylglucosaminidase by *N*-acetylgalactosaminides and inhibition of *N*-acetylgalactosaminidase by *N*-acetylglucosaminides. The second is of one site with competition, together with a direct displacement mechanism of one substrate by another with special assumptions for the velocity constants for the displacement.

6. Further experimental evidence of a relationship between Michaelis and inhibitor constants of two competing substrates supports the second scheme.

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Studies on Glucosaminidase

6. *N*-ACETYL-β-GLUCOSAMINIDASE AND *N*-ACETYL-β-GALACTOSAMINIDASE ACTIVITIES OF A VARIETY OF ENZYME PREPARATIONS

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A survey of a large number of enzyme preparations for *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities has been undertaken to establish whether the close association of these activities which had been found with a testicular preparation (Woollen, Heyworth & Walker, 1961) and a kidney preparation (Walker, Woollen & Heyworth, 1961) was general. No enzyme source was discovered which had one activity and not the other. The two activities were never additive when both substrates were present together, so there was no evidence of specific sites.

MATERIALS AND METHODS

Enzyme preparations

Human pregnancy sera. These were obtained from one subject at 6 and 24 weeks of pregnancy.

Pig epididymis. Crude and purified preparations corresponding to the 'best bit', stages 2 and 7*b*, of specific activity 194 000 and 3 280 000 (Findlay & Levvy, 1960) were diluted in 0·1% (w/v) crystallized bovine albumin (Armour Ltd.) for assay.

Ram testis. The freeze-dried extract (Benger Ltd., Holmes Chapel, Cheshire) was made 0·1% (w/v) in 0·1% (w/v) crystallized bovine albumin (Woollen *et al.* 1961).

Rat tissues. Fresh tissues were weighed and ground in water in a glass homogenizer and made to a final concentration (w/v) of 10% for liver, spleen and testis, 2% for kidney and salivary gland (submaxillary and sublingual) and 1% for epididymis, in 0·05M-sodium citrate–citric acid buffer, pH 4·5. Clear supernatants were obtained by centrifuging at 1500*g* for 10 min. Such supernatants have 80–90% of the *N*-acetylglucosaminidase activity of whole homogenates.

Viper venom. Russell viper venom (Wellcome Research Laboratories), 1% (w/v) in water, was used.

Leech. Hirudo medicinalis specimens were killed with chloroform, and were weighed and homogenized in water. The extract (10%, w/v) was centrifuged at 1500*g* for 10 min. to obtain a clear supernatant. A 5% (w/v) extract of leech heads was similarly prepared.

Snail and limpet preparations. The digestive tract of *Helix pomatia* and the digestive gland of *Patella vulgata* (both killed with chloroform) were weighed, homogenized with water and diluted to give concentrations (w/v) of 4% and 10% respectively.

Locust-crop fluid. This was obtained from adult *Schistocerca gregaria* at the Anti-Locust Research Centre, London.

Sweet-almond emulsin. Partially de-fatted sweet-almond meal (5 g.) was shaken at 4° with 100 ml. of 0·05 M-citrate buffer, pH 4·5, for 15 min. and then allowed to stand for 2 hr. at 4° and filtered to give a clear extract.

Micro-organisms

Aspergillus oryzae. This preparation was a 0·1% (w/v) solution in water of Hydrolase Mixture (C. F. Boehringer und Söhne, Mannheim, Germany).

Proteus vulgaris. A freeze-dried extract (Dodgson, Lloyd & Spencer, 1957; preparation B) was made 1% (w/v) in water.

Trichomonas foetus. A freeze-dried extract (Watkins, 1959) was made 0·05% (w/v) in 1% (w/v) crystallized bovine albumin.

Lycoperdon pyriforma *and* Lycoperdon giganteum *extracts.* These were prepared from the fungi according to Tracey (1955).

Clostridium perfringens (N.C.T.C. 2836). A 10 hr. culture in Robertson's cooked-meat medium (see Mackie & McCartney, 1942) was subcultured into 250 ml. of the same medium and incubated for 12 hr. in screw-capped bottles at 37°. The cultures were filtered through muslin to remove meat particles, centrifuged at 14 000*g* for 30 min., resuspended in 0·9% sodium chloride and again centrifuged. The cells were disrupted with polishing γ-alumina (McIlwain, 1948) and the alumina was eluted with 0·1 M-sodium phosphate buffer, pH 7·4, containing 10% (w/v) of ammonium sulphate. This solution was dialysed against 1% (w/v) sodium chloride for 48 hr. and, for some investigations, made 1% (w/v) with crystallized bovine albumin (see Results section).

Cultures were grown at 37° for 16 hr. of *Alcaligenes faecalis, Bacillus megaterium, Pseudomonas aeruginosa, Staphylococcus pyogenes* and *Enterococcus* on digest broth; of *Escherichia coli* in peptone water; of *Streptococcus pyogenes* (Lancefield group A) in glucose broth; of *Clostridium sporogenes* in Robertson's cooked-meat medium; and for 48 hr. of *Pneumococcus* type 3 in horse-serum broth. After centrifuging the cells were suspended in 10 ml. of 0·9% sodium chloride.

Table 1. N-*Acetylglucosaminidase and* N-*acetylgalactosaminidase activities*

Substrates were 1·75 mM-*p*-nitrophenyl *N*-acetylglucosaminide or 1·75 mM-*p*-nitrophenyl *N*-acetylgalactosaminide or both. For other conditions see the Methods section. Activities are expressed as μg. of *p*-nitrophenol liberated/unit of enzyme source indicated.

Substrates	Activities: *N*-Acetylglucosaminide	Activities: *N*-Acetylgalactosaminide	Activities: *N*-Acetylglucosaminide + *N*-acetylgalactosaminide	Units of enzyme source
Enzyme preparations:				
Human pregnancy serum*				
6 weeks	47	6	14	ml.
24 weeks	159	18	44	ml.
Pig epididymis				
Stage 2	$0{\cdot}31 \times 10^6$	$0{\cdot}05 \times 10^6$	$0{\cdot}12 \times 10^6$	ml.
Stage 7*b*	$23{\cdot}10 \times 10^6$	$3{\cdot}75 \times 10^6$	$7{\cdot}95 \times 10^6$	ml.
Ram testis	314	68	144	mg.
Rat-tissue extracts				
Kidney	16·0	1·9	4·0	mg.
Liver	5·3	0·8	1·9	mg.
Spleen	4·8	0·8	1·5	mg.
Testis	2·8	0·4	1·1	mg.
Salivary gland	22·8	2·3	5·1	mg.
Epididymis	103·8	10·8	25·2	mg.
Viper venom	19·3	2·9	5·6	mg.
Hirudo medicinalis				
Whole	19·4	10·5	13·7	mg.
Head	15·5	7·1	9·5	mg.
Helix pomatia digestive tract	72·7	33·1	56·0	mg.
Patella vulgata digestive gland	13·4	5·4	15·1	mg.
Schistocerca gregaria crop fluid	$64{\cdot}6 \times 10^3$	$26{\cdot}0 \times 10^3$	$40{\cdot}8 \times 10^3$	ml.
Sweet-almond emulsin	8·2	5·4	5·6	mg.
Aspergillus oryzae	260	221	214	mg.
Proteus vulgaris	31·4	4·7	10·3	mg.
Trichomonas foetus	472	352	414	mg.
Lycoperdon pyriforma	460	320	410	ml.
L. giganteum	520	790	460	ml.
Clostridium perfringens†	224	12	216	ml.
Pneumococcus, type 3	348	45	325	ml.
Enterococcus	180	7	172	ml.

* At pH 4·3 (Walker *et al.* 1960).

† At pH 5·05

Substrates

p-Nitrophenyl *N*-acetyl-β-D-glucosaminide and the corresponding galactosaminide were prepared according to Leaback & Walker (1957) and Heyworth, Leaback & Walker (1959) respectively.

Estimation of enzyme activities

The method is described by Woollen *et al.* (1961). Except where indicated, the enzyme solutions were incubated with substrate(s) in a total volume of 1 ml. for 30 min. in 0·05 M-citrate buffer, pH 4·5, at 37°.

Antitoxin

The *C. perfringens* type A antiserum was from The Wellcome Research Laboratories (reference EX 1863).

RESULTS

N-Acetylglucosaminidase and N-acetylgalactosaminidase activities of the various enzyme preparations. The amount of hydrolysis was measured in the presence of 1·75 mM-*p*-nitrophenyl *N*-acetylglucosaminide or 1·75 mM-*p*-nitrophenyl *N*-acetylgalactosaminide or in mixtures containing these concentrations of both substrates (Table 1). *N*-Acetylglucosaminidase activity was always accompanied by *N*-acetylgalactosaminidase activity, although there was a wide variation in the ratio of these activities (1–26) in the different sources under the conditions used. There was no apparent correlation between the ratios and the biological origins of the enzyme preparations.

With human pregnancy serum the *N*-acetylgalactosaminidase activity appeared to be increasing during pregnancy parallel with *N*-acetylglucosaminidase (Walker, Woollen & Pugh, 1960). Similarly, with the purified preparations of pig epididymis (Findlay & Levvy, 1960), the relative *N*-acetylglucosaminidase activity to *N*-acetylgalactosaminidase activity remained constant over the 17-fold increase in specific activity.

The relative *N*-acetylglucosaminidase activities of the various rat tissues are in agreement with those found by Conchie, Findlay & Levvy (1959) with whole homogenates.

The *N*-acetylgalactosaminidase activity of *T. foetus* was greater, relative to *N*-acetylglucosaminidase, than that found at pH 7 by Watkins (1959).

In every case the amount of hydrolysis in mixtures of the two substrates was less than the sum of those obtained with each substrate individually.

No activity was detected with either substrate under the conditions used with *P. aeruginosa*, *C. sporogenes*, *Alcaligenes faecalis*, *B. megaterium*, *Strep. pyogenes*, *Staphylococcus pyrogenes* or *E. coli*.

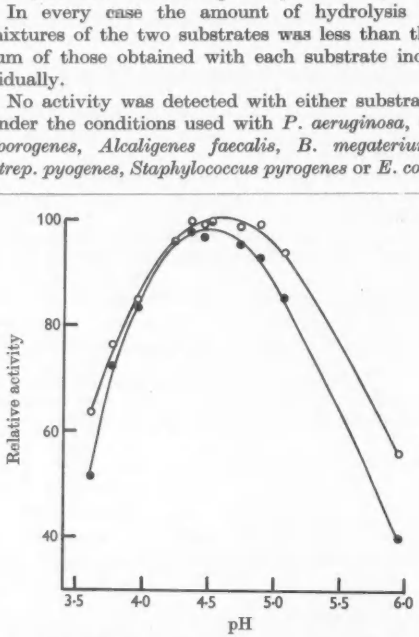

Fig. 1. Effect of pH on the emulsin activities. For 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide and 0·1% (w/v) of enzyme (○) a relative activity of 100 corresponds to 10·5 μg. of *p*-nitrophenol liberated/tube. For 2·2 mM-*p*-nitrophenyl *N*-acetylgalactosaminide and 0·3% (w/v) of enzyme (●) a relative activity of 100 corresponds to 23·3 μg. of *p*-nitrophenol liberated/tube. For other conditions see the Methods section.

Table 2. *Ammonium sulphate fractionation and partial heat inactivation of emulsin*

Fractions were precipitated between the indicated limits of percentage saturation with ammonium sulphate. Preincubation was carried out at 56° in the absence of substrate in 0·05 M-citrate buffer, pH 4·5. For the assays the substrate concentration was 2·2 mM. For other conditions see the Methods section.

	N-Acetylglucosaminidase (% of untreated preparation)		Ratio *N*-acetylglucosaminidase: *N*-acetylgalactosaminidase	
Period of preincubation (min.) ...	0	5	0	5
Enzyme preparation:				
Untreated	100*	66·0	1·7	2·4
$(NH_4)_2SO_4$ fractions				
0–40%	4·3	2·3	1·2	1·9
40–50%	4·4	2·4	1·1	2·0
50–60%	40·1	26·4	1·7	2·3

* 60·4 μg. of *p*-nitrophenol liberated/tube.

N-Acetylhexosaminidase activities of sweet-almond emulsin. A kinetic study of the two activities in emulsin was initiated like the studies on a testicular preparation (Woollen *et al.* 1961) and a kidney extract (Walker *et al.* 1961). The effect of pH was studied in 0·05 M-citrate buffer (Fig. 1). At pH 4·5 both enzyme activities were proportional to enzyme concentration over a fourfold range and also to time of incubation over 1 hr. Although substrate competition was demonstrated, further experiments showed differences from the testis and kidney preparations. Fractionation with ammonium sulphate and partial heat inactivation of these fractions as described by Woollen *et al.* (1961) both led to variations in the ratio of *N*-acetylglucosaminidase : *N*-acetylgalactosaminidase activities (Table 2). This may indicate the presence of two or more enzyme proteins of unknown specificity.

N-Acetylhexosaminidase activity of Clostridium perfringens. An initial inability to detect *N*-acetylgalactosaminidase activity in these preparations led to an investigation of optimum conditions. Experience with a testicular preparation had shown instability of *N*-acetylglucosaminidase and a loss of specific activity on dilution of the partially purified enzyme, both of which were overcome in the presence of 0·1% (w/v) crystallized bovine albumin. A similar loss of specific activity on dilution was observed with the *C. perfringens* preparation. The presence of albumin was found to increase the rate of hydrolysis of *p*-nitrophenyl-*N*-acetylglucosaminide (Fig. 2) and in a final concentration of 0·1% (w/v) gave proportionality between activity and enzyme concentration. Activity was also proportional to time of incubation over 45 min. In the presence of 0·1% of albumin there was an optimum pH of 5·05 in 0·05 M-citrate buffer (Fig. 3) and *N*-acetylgalactosaminidase activity was found under these optimum conditions (Table 1). Under these conditions *C. perfringens* antitoxin was shown to be a non-competitive inhibitor of *N*-acetylglucosaminidase activity.

DISCUSSION

It was concluded from the failure of all attempts to separate the *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities in ram testis (Woollen *et al.* 1961) and rat kidney (Walker *et al.* 1961) and from the detailed kinetic studies carried out with those preparations that both activities were associated with a single active site on the same enzyme protein. In the present paper it was shown that every enzyme preparation that had *N*-acetyl-

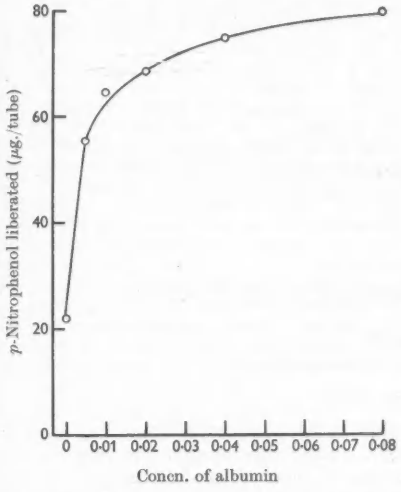

Fig. 2. Effect of albumin concentration on *C. perfringens* *N*-acetylglucosaminidase activity in 0·05 M-citrate buffer, pH 5·05, with 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide. For other conditions see the Methods section.

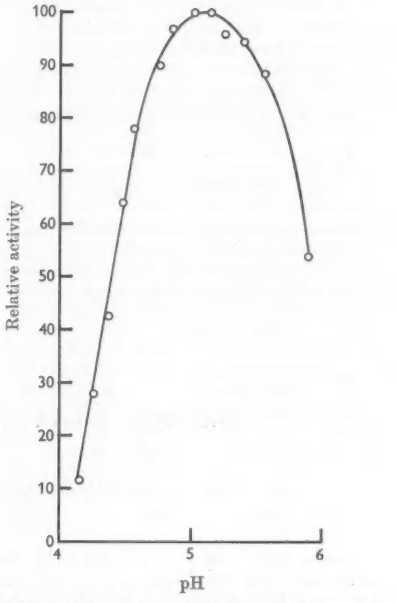

Fig. 3. Effect of pH on *N*-acetylglucosaminidase activity of *C. perfringens* with 0·05 M-citrate buffer and 3·6 mM-*p*-nitrophenyl *N*-acetylglucosaminide. For other conditions see the Methods section. A relative activity of 100 corresponds to 51·8 μg. of *p*-nitrophenol liberated/tube.

glucosaminidase activity also had *N*-acetylgalactosaminidase activity and that in each case the total hydrolysis in mixtures of the two substrates was less than the sum of those of the individual substrates. There was therefore no evidence of a specific site for either substrate.

It cannot, however, be concluded from these results that the same enzyme is responsible for the hydrolysis of both substrates in every case. Fractionation and differential inactivation of the activities in emulsin indicated the presence of more than one active protein. More detailed investigations are required before any further conclusion can be drawn about the relationship of *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase activity in each case.

The increased *N*-acetylglucosaminidase activity in the *C. perfringens* enzyme preparation in the presence of albumin appears to be another example of protection by surface-active agents against inactivation in purified enzymes. This has been described for *N*-acetylglucosaminidase (Woollen *et al.* 1961), for β-glucuronidase (Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954), for acid phosphatase (Tsuboi & Hudson, 1955) and cellulase (Myers & Northcote, 1959).

C. perfringens antitoxin has been shown to inhibit the hyaluronidase (McClean & Hale, 1941) and the lecithinase (Macfarlane & Knight, 1941) of this organism, the latter non-competitively (Cinader, 1953). The antitoxin has been shown here to act non-competitively with *N*-acetylglucosaminidase.

SUMMARY

1. The *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities of a wide variety of enzyme preparations have been measured.

2. Where *N*-acetylglucosaminidase was detected it was accompanied by *N*-acetylgalactosaminidase but the ratios of the two activities covered a wide range.

3. Experiments with mixed substrates did not indicate a specific site for either activity in any of the preparations.

We gratefully acknowledge the gifts of viper venom from Dr H. Proom and of *C. perfringens* antiserum from Dr G. H. Warrack of The Wellcome Research Laboratories and of various enzyme sources from Dr W. M. Watkins, Dr K. S. Dodgson, Benger Ltd., the Anti-Locust Research Centre and the Pathology department of this Institute. The work was assisted by grants from the Research Fund of the University of London and the Nuffield Foundation.

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The Metabolism of Protocatechuic Acid by a Vibrio

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Recent work with micro-organisms has suggested that the mechanisms of biological ring-fission may be considerably more diverse than was originally suspected. Not only has a new mode of fission of the benzene nucleus been reported (Dagley & Stopher, 1959; Trippett, Stopher & Dagley, 1960), but additional pathways have been formulated whereby the aliphatic products resulting from ring-fission are channelled into terminal oxidation cycles (Ribbons & Evans, 1959). This paper describes the metabolism of protocatechuic acid by *Vibrio* O1 (Happold & Key, 1932)

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and, in particular, the participation in this scheme of a lactone previously found only as an intermediate in the bacterial degradation of catechol.

EXPERIMENTAL

Organisms. Vibrio O1 (N.C.I.B. 8250) was maintained by monthly subculture both on nutrient agar and slopes of the defined medium (see below) solidified with agar. *Neurospora crassa* (wild-type) (SY4a) was obtained from Professor W. C. Evans, University College of North Wales, Bangor, Caernarvonshire. Mutants of this mould, Y7655a and Y7655–10575a, with growth requirements for aromatic compounds, were obtained from Professor E. L. Tatum (Tatum & Gross, 1956). Each of the moulds was maintained on standard maltose–peptone medium [maltose (2%, w/v); peptone (1%, w/v); pH 6·0] and also on minimal medium (Ryan, Beadle & Tatum, 1943) with glucose (1%, w/v) as the carbon source, and for the mutants with the aromatic supplement (Gross, Gafford & Tatum, 1956). Vanillic acid (0·02%, w/v) was added as an inducer of protocatechuic acid oxidase in the wild-type strain when required. *Nocardia erythropolis* CA4 was the strain isolated from cultures enriched with *p*-nitrobenzoic acid (Cain, 1958).

Growth conditions. The vibrio was grown for 24–50 hr. with forced aeration in 10 l. flasks, containing 9 l. of defined medium, after inoculation with 50 ml. of a culture grown overnight in the same medium. This medium contained (g./l.): K_2HPO_4, 2·0; $(NH_4)_2SO_4$, 1·0; $MgSO_4,7H_2O$, 0·1; *p*-hydroxybenzoic acid, 1·0; the pH was adjusted to 7·0–7·2 with NaOH. Growth under these conditions gave cells strongly adapted to oxidize protocatechuic acid. Unadapted cells were grown either in a defined medium where succinic acid (1 g./l.) replaced the aromatic substrate or in a nutrient medium containing (g./l.); Bacto peptone, 2·0; Lab-Lemco, 0·5; the pH was adjusted to 7·0–7·2. Cells were harvested by continuous centrifuging in an Alfa-Laval separator (Alfa-Laval Ltd., Brentford, Middlesex).

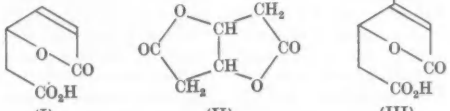
Cell-free extracts and heat-treatment. Extracts of the organism were prepared, either by ultrasonic irradiation for 20–30 min. with the M.S.E. Mullard 60w apparatus at 20 kcyc./sec. with the cell suspension cooled in melting ice, or by crushing in a Hughes press (Hughes, 1951) without abrasive. The crush was taken up in 0·05M-phosphate buffer, cell debris and particulate material were removed by centrifuging at 16 000 *g* for 1 hr., and the clear supernatant liquid (usually 15–25 mg. of protein/ml.) was used as a crude extract. This supernatant contained all the enzymes required to convert protocatechuic acid into β-oxoadipic acid. For heat-treated extracts, samples of about 10 ml. of the extract in 0·05M-phosphate buffer were rapidly heated to 55° in a water bath (with the thermometer agitating the extract), held at 55±0·5° for 15 min., and then cooled in melting ice. The flocculent coagulated protein was usually removed by centrifuging for 30 min. at 16 000 *g*. Appreciable loss of activity occurred if the heat-treatment was performed on extracts made without buffer.

Chemicals. All melting points are uncorrected. β-Oxoadipic acid, m.p. 119°, was synthesized by the method of Bardhan (1936). β-Carboxymuconic acid (tri sodium salt) was prepared biologically from protocatechuic acid by using the purified protocatechuic acid oxidase from *Nocardia erythropolis* (Cain & Cartwright, 1960). Generous gifts of this material were also received from Professor W. C. Evans, Bangor, and Dr D. L. McDonald, Berkeley. The purest available preparations contained 93% of active isomer by spectrophotometric and enzymic assays. *cis–cis*-Muconic acid, m.p. 195°, was prepared by peracetic acid oxidation of pure phenol (Elvidge *et al.* 1950). Gifts of synthetic 4-hydroxyhex-2-enedioic 1→4-lactone (muconolactone) (I), m.p. 109°, and 2:6-dioxa-3:7-dioxo*bicyclo*-[3:3:0]octane (dilactone) (II), m.p. 127°, were received from Professor Evans.

The unnatural (−)-isomer of muconolactone, m.p. 79° was prepared by incubation of the synthetic (racemic) material with a washed cell suspension of the vibrio, grown on *p*-hydroxybenzoic acid, until half the substrate had disappeared. The use of washed cells, which degrade only the natural isomer completely, is preferable to resolution with extracts or the purified delactonizing enzyme, since it avoids accumulation of β-oxoadipic acid as the end product of the reaction.

3-Carboxy-4-hydroxyhex-2-enedioic 1→4-lactone (β-carboxymuconolactone; III) was prepared from the corresponding acid by modification of the method of Gross *et al.* (1956). The mycelia of the wild-type *Neurospora* (SY4a), obtained after 3 or 4 days growth with gentle shaking at 30° in horizontal Roux flasks containing 100 ml. of glucose minimal medium + vanillic acid, were harvested by filtration, washed three times, freeze-dried and stored at −14° till required.

The freeze-dried mycelium (1·5 g.) was moistened with 2–3 ml. of water in a precooled mortar and vigorously ground with washed, finely powdered glass. The crushed mycelium was extracted with 5–10 ml. of water for 15–30 min., centrifuged at low speed to remove cell debris and dialysed against several changes of distilled water for 18–24 hr. The dialysed material was centrifuged at high speed (20 000 *g*, 40 min.), and the supernatant solution (about 12 ml.) was made 2 mM with respect to phosphate at pH 6·2 and then treated with 0·1 vol. of calcium phosphate gel (Keilin & Hartree, 1938) for 30 min. with gentle agitation. After removal of the gel by centrifuging, the supernatant was brought to pH 6·9 with 0·1M-phosphate buffer, pH 7·8, and the gel-treatment repeated; 75–80% of the



(I) (II) (III)

lactonizing activity remained in the supernatant. This dialysis and gel-treatment sequence was repeated until spectrophotometric assay at 225 and 270 mμ with β-carboxymuconic acid as substrate indicated the absence of delactonizing enzyme. β-Carboxymuconolactone was then prepared on a small scale from β-carboxymuconic acid essentially as described by Gross *et al.* (1956) except that the reaction was performed in 0·1 M-phosphate buffer, pH 7·0, to avoid constant pH adjustment. The lactone was extracted from the aqueous solution by continuous ether extraction for 4 days or until the lactone concentration in the aqueous layer was minimal. The product (25 mg., m.p. 163–164°) agreed in properties with those of β-carboxymuconolactone, described by Gross *et al.* (1956). All other organic chemicals were commercial samples generally recrystallized except when used for bulk growth media.

Estimations. Protocatechuic acid and β-oxoadipic acid were estimated manometrically, the former by O_2 uptake with the purified *Nocardia* oxidase (Cain & Cartwright, 1960), the latter by catalytic decarboxylation at 30°, pH 4·0, with 0·1 M-aminoantipyrine (Sistrom & Stanier, 1953). Protocatechuic acid, β-carboxymuconic acid and β-carboxymuconolactone were estimated at 290, 270 and 225 mμ on a Unicam SP. 500 spectrophotometer. At these wavelengths and pH 7·0, the molar extinctions are: protocatechuic acid: $\epsilon_{270\ m\mu}$ 2730, $\epsilon_{290\ m\mu}$ 3890; β-carboxymuconic acid: $\epsilon_{225\ m\mu}$ 9400, $\epsilon_{270\ m\mu}$ 6400, $\epsilon_{290\ m\mu}$ 1590; β-carboxymuconolactone: $\epsilon_{225\ m\mu}$ 8900; $\epsilon_{270\ m\mu}$ 570. Muconolactone and the dilactone were estimated colorimetrically as their hydroxamic acid derivatives. The dilactone forms a hydroxamate under the acid conditions (pH 6·4) described by Lipmann & Tuttle (1945). At this pH, muconolactone fails to react. Both lactones, however, form hydroxamates under the alkaline conditions (pH 9·0) of Hestrin's (1949) method, so that either can be determined in the presence of the other. The final procedure adopted was as follows.

(*a*) Acid hydroxamate (dilactone). To 1 ml. of the test solution was added 1 ml. of hydroxylamine solution (equal volumes of 4 M-hydroxylamine hydrochloride and 3·5 M-NaOH) and 1 ml. of acetate buffer (0·05 M, pH 5·4), and the solution kept for 10 min. Then 1 ml. of HCl (conc. acid diluted 1 in 3), 1 ml. of trichloroacetic acid (10%, w/v) and 1 ml. of $FeCl_3,6H_2O$ (5·0% in 0·1 N-HCl) were added successively and the red–purple colour was read at 500 mμ, after 10 min., on a Unicam SP. 600 spectrophotometer.

(*b*) Alkaline hydroxamate (muconolactone). The procedure was identical, but the hydroxylamine solution contained equal volumes of 2 M-hydroxylamine hydrochloride and 3·5 M-NaOH, water replaced the acetate buffer, and the HCl was made by diluting the conc. acid 1 in 4. Precipitated protein was removed by centrifuging at 3000 *g* for 5 min. in both methods. Standards were prepared with synthetic specimens of both dilactone and muconolactone; estimations on known amounts under normal experimental conditions have given excellent agreement.

Protein was estimated by the biuret method of Gornall, Bardawill & David (1949) with bovine albumin (Fraction V, Armour Laboratories) as a standard, or by the phenol method of Lowry, Rosebrough, Farr & Randall (1951).

Buffers. Incubations were carried out either in Sørensen Na_2HPO_4–KH_2PO_4 buffer or in 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer. The molarity and pH used are indicated in the text.

Enzymic assays. In the nomenclature of these enzymes, the system employed by Sistrom & Stainer (1954) has been used: 'lactonizing enzyme' describes the system synthesizing a lactone and 'delactonizing enzyme', one which degrades it. Protocatechuic acid oxidase and the lactonizing enzyme of *Vibrio* O1 were assayed spectrophotometrically by disappearance of their respective substrates. The assay system contained: substrate, 0·5 μmole; tris–HCl buffer, 100 μmoles, pH 7·4; up to 0·2 mg. of enzyme protein; water to a total volume of 3 ml. Cuvettes of light-path 10 mm. were used with a constant-temperature cell carrier at 30°. The delactonizing enzyme was assayed either by disappearance of substrate (muconolactone) at 30°, pH 7·4, or because of the smaller quantities of substrate required for an equally accurate assay, by manometric estimation of the β-oxoadipic acid produced. Both methods gave similar results in trial runs. The reaction mixture in Warburg flasks for manometry contained: (+)-muconolactone, 10 μmoles; tris–HCl buffer, 100 μmoles, pH 7·4; about 3–4 mg. of enzyme protein; water to a total volume of 3·0 ml. At given times (after incubation for 15, 20 or 30 min. at 30°) the reaction was stopped by the addition of 0·1 ml. of acetic acid, and the β-oxoadipic acid formed was estimated by decarboxylation. A linear relationship between time and β-oxoadipic acid produced was established.

Chromatography. All solvent proportions are by volume. The aliphatic acids were run in solvent *A* [ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (80:4:16)] and were detected by spraying with ethanolic bromothymol blue (0·05%, w/v) made just alkaline with dil. NH_3 soln. This solvent gave very satisfactory separation of *cis–cis*-muconic acid and β-carboxymuconic acids from each other and from their respective lactones, which, because of their free carboxyl groups, also react as acids. Muconolactone and the dilactone were run as their hydroxamates in solvent *B* [pyridine–acetic acid–water (50:35:15)] or, as the unchanged lactone, in both solvent *C* [pyridine–ethyl acetate–water (1:1:1)] and solvent *D* [diethyl ether–benzene–formic acid (98%, w/v)–water (70:30:14:10)]. When run as the hydroxamates, the lactones were detected with $FeCl_3$ solution (5% in 0·1 N-HCl diluted 1 in 4 with ethanol) and showed up as reddish-purple spots. More intensely coloured and clearly defined spots were obtained by forming the hydroxamates on the paper after running as the unchanged lactone. The procedure adopted employed the reagents for alkaline hydroxamate estimation (except the water) applied in the described order (after diluting each reagent 1 in 4 with ethanol) by means of a fine spray to avoid over-wetting the paper with resulting streaking of the spots. It was essential, after forming the hydroxamate on the paper under alkaline conditions, to spray with sufficient ethanolic HCl to render the chromatogram completely acidic. Without this precaution, the lactone spots were disguised by $Fe(OH)_3$ formation when ethanolic $FeCl_3$ was applied. Dinitrophenylhydrazones were run in solvent *E* [2-methylbutan-2-ol–propan-1-ol–aq. NH_3 solution (sp.gr. 0·88) (65:5:30)], and the chromatograms examined under ultraviolet light or sprayed with 5% (w/v) Na_2CO_3. All chromatograms were run by the descending method on Whatman no. 542 paper.

Manometric procedures. Uptake of O_2 and evolution of CO_2 were followed by the conventional manometric procedures, (Umbreit, Burris & Stauffer, 1949). Evolution of

CO_2 was often measured under anaerobic conditions, in which case the flask and manometer were gassed with O_2-free N_2 for 15 min. before temperature equilibration.

RESULTS

Conversion of protocatechuic acid into β-oxoadipic acid

p-Hydroxybenzoic acid is a well-known inducer of protocatechuic acid oxidase in many bacteria (Stanier & Ingraham, 1954; Durham, 1956; Cartwright & Cain, 1959), but the nature of the degradation products of protocatechuate has not previously been investigated in this vibrio, although it has been used in many experiments with aromatic compounds. Crude extracts of 24 hr.-old cells grown on *p*-hydroxybenzoic acid rapidly oxidized protocatechuic acid, but not catechol, with the theoretical O_2 uptake and production of a material giving a strong Rothera-positive reaction (Table 1). This material was isolated from a larger-scale experiment and identified by melting point (117–118°) and mixed melting point (119°) with authentic β-oxoadipic acid; the identity was confirmed by co-chromatography as the dinitrophenylhydrazone in solvent *E*.

Oxidation of protocatechuic acid to β-carboxymuconic acid

McDonald, Stanier & Ingraham (1954) prepared from a *Pseudomonas* sp. a protocatechuic acid oxidase which catalysed the conversion of protocatechuic acid into β-carboxymuconic acid. This latter compound has since been found as the primary ring-opening product in protocatechuic acid oxidation by *Neurospora crassa* (Gross *et al.* 1956), *Nocardia erythropolis* (Cain & Cartwright, 1960) and a pseudomonad isolated from phthalic acid enrichments (Ribbons & Evans, 1960). It was likely therefore that the vibrio also possessed the enzyme, although no evidence of β-carboxymuconic acid accumulation was detectable by spectrophotometric analysis of the course of protocatechuic acid oxidation with crude extracts. With the following scheme, however, a protocatechuic acid oxidase, free from enzymes degrading β-carboxymuconic acid, was obtained and was used to follow accumulation of this product.

A Hughes-press extract of cells grown with *p*-hydroxybenzoic acid was taken up in 0·05 M-phosphate buffer and heat-treated for 16 min. at 55°. This removed considerable protein without appreciably affecting the activity of the oxidase. The coagulated protein was centrifuged off, and the supernatant solution dialysed for 24 hr. against 1 mM-ethylenediaminetetra-acetate (EDTA) at pH 7·0 and then against two changes of distilled water for 48 hr., all at 4° in a cold room. The dialysed material was treated with neutral, saturated $(NH_4)_2SO_4$ solution; all the activity was precipitated in the 0–0·45 saturation range. This fraction was taken up in water, dialysed to remove all salts, brought to pH 5·5 with 0·05 M-acetate buffer and treated twice with 0·3 vol. of calcium phosphate gel for 15 min.

Table 1. *Oxidation of protocatechuic acid to β-oxoadipic acid by crude extracts*

Each Warburg flask contained: phosphate buffer, pH 7·0, 150 μmoles; substrate as shown; enzyme protein, 3·8 mg.; KOH, 0·2 ml. in centre well; water to a final volume of 3 ml. Temp., 30°; gas phase, air. At completion of O_2 uptake, the reaction was terminated by addition of 0·1 ml. of acetic acid, the KOH swabbed from centre well, and 0·4 ml. of 0·1 M-4-aminoantipyrine added to side arm. After temperature re-equilibration, this catalyst was tipped and β-oxoadipic acid estimated by the resulting evolution of CO_2. Results are corrected for gas changes in the absence of substrate.

Protocatechuic acid added (μmoles)	O_2 uptake (μmoles)	β-Oxoadipic acid produced (μmoles)
2·0	1·8	1·7
3·0	2·8	2·7
5·0	4·9	4·7

Table 2. *Purification of protocatechuic acid oxidase from* Vibrio O1

The unit of activity is defined as that amount of enzyme causing an extinction decrement of 0·01/min. at 290 mμ under the following assay conditions: phosphate buffer, 200 μmoles, pH 7·0; protocatechuic acid, 0·5 μmole; enzyme, 0·3 ml. Fe^{2+} ions (0·1 ml.), to give a final concentration of 0·33 mM, were incubated with the enzyme for 20 min. before addition of substrate. Supplementation with Fe^{2+} ions was later found unnecessary.

Material	Activity (units/ml.)	Protein (mg./ml.)	Specific activity (units/mg. of protein)
Crude extract	27·0	10·80	2·5
Heat-treated and dialysed extract	26·0	7·50	3·5
$(NH_4)_2SO_4$ fraction, 0–45 % saturation	18·5	2·20	8·4
1st gel eluate, pH 7·8, 0·1 M-buffer	5·5	0·28	19·5
2nd gel eluate:			
(i) pH 6·4, 0·1 M-buffer	0·06	0·05	1·2
(ii) pH 7·2, 0·1 M-buffer	4·1	0·17	24·0*

* This fraction is devoid of lactonizing activity.

with gentle agitation. All the oxidase was absorbed on the gel, which was washed with water. The oxidase could be eluted with 0·1 M-phosphate buffer at pH 7·8, but spectrophotometric analysis showed that lactonizing enzyme was still present. The eluate was therefore dialysed, brought to pH 5·5 and readsorbed on more gel. The gel was washed with water and eluted with 0·05 M-phosphate buffer, pH 6·4; the eluate, containing inactive protein, was discarded and the gel further eluted with 0·1 M-buffer at pH 6·4, again removing enzyme of low activity. The oxidase was finally eluted with 0·1 M-phosphate buffer at pH 7·2 or higher. No attempt was made to obtain a high specific activity or maintain a large proportion of the total enzyme units. Table 2 shows that the overall purification is small but that the final product is free of the enzymes which degrade β-carboxymuconic acid and could be used to accumulate this compound from protocatechuic acid.

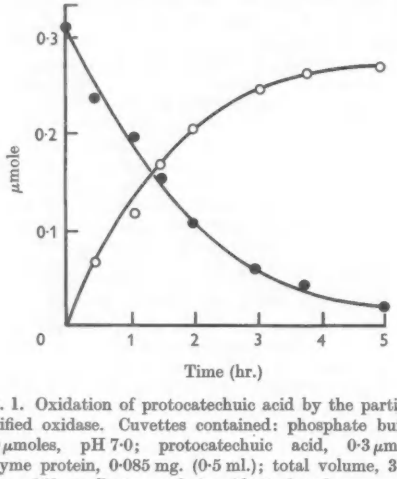


Fig. 1. Oxidation of protocatechuic acid by the partially purified oxidase. Cuvettes contained: phosphate buffer, 200 μmoles, pH 7·0; protocatechuic acid, 0·3 μmole; enzyme protein, 0·085 mg. (0·5 ml.); total volume, 3 ml. Temp., 30°. ●, Protocatechuic acid; ○, β-carboxymuconic acid.

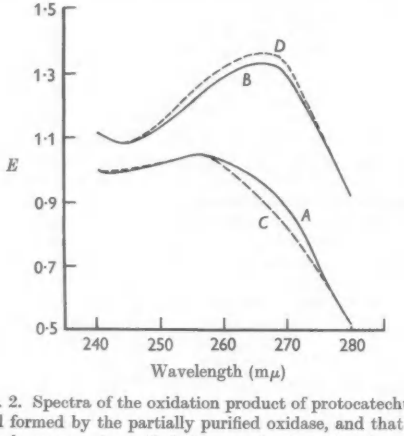


Fig. 2. Spectra of the oxidation product of protocatechuic acid formed by the partially purified oxidase, and that of β-carboxymuconic acid before, and after, heating for 30 min. at 100°. —, Oxidation product before (*A*) and after (*B*) heating; - - - -, authentic β-carboxymuconic acid before (*C*) and after (*D*) heating.

Fig. 1 shows the course of the oxidation followed spectrophotometrically and Fig. 2 the spectrum of the reaction product. This spectrum is identical with that of authentic β-carboxymuconic acid in all respects including the shift in position and increase in extinction of the absorption peak on boiling for 30 min. Manometric experiments showed that for every mole of protocatechuate oxidized, 1 mole of O_2 was consumed, no CO_2 was evolved and 1 mole of β-carboxymuconic acid, estimated spectrophotometrically, was produced by the purified oxidase; the reaction was essentially irreversible. Chromatography of the reaction mixtures at the completion of such oxidations showed a compound which moved an identical distance to authentic β-carboxymuconic acid and failed to separate from mixtures with it in solvent *A*.

Lactone production by heat-treated extracts of the vibrio

The enzymic conversion of protocatechuic acid into a compound reacting as a lactone was first observed during a class experiment designed by Dr S. Dagley to demonstrate accumulation of β-oxoadipic acid. A crude bacterial extract, which had been stored with intermittent freezing and thawing, catalysed the oxidation of protocatechuic acid, but β-oxoadipate was not produced; a lactone accumulated instead. The reason for this was not clear, and, accordingly, extracts showing no such lactonizing activity, freshly prepared from adapted cells, were treated in various ways by the author to reproduce similar behaviour. The most significant results were those obtained by heat-treatment of extracts for various periods. When extracts were maintained at 55° for 15 min., the delactonizing enzymes were completely destroyed, whereas those catalysing formation of the lactone retained their activity (Fig. 3). The lactone which accumulated in such incubations fulfils the requirement of a true intermediate since addition of crude extract led to its rapid disappearance (Fig. 4). The lactone formed in these incubations gave a spectrum similar to that of authentic muconolactone (Fig. 5, $\epsilon_{max.}$ at 207 mμ) and ran as muconolactone in chromatograms with solvents *C* and *D*. No evidence for the anticipated carboxymuconolactone (ϵ_{max} at 216 mμ) was found, nor did the vibrio produce any lactone forming a hydroxamate under acid conditions (e.g. the dilactone).

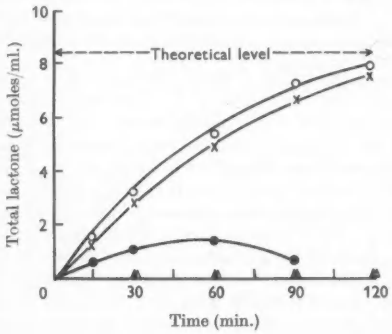


Fig. 3. Lactone accumulation as a result of heat-treating crude extracts of the vibrio. The incubations were carried out in boiling tubes vigorously shaken at 270 strokes/min., 5 cm. throw, aerobically. Tubes contained: phosphate buffer, 1 m-mole, pH 7·0; sodium protocatechuate, 100 μmoles; protein extract, 12·8 mg.; total volume, 12·0 ml. Temp., 30°. Control tubes contained (i) enzyme boiled for 10 min. and (ii) no substrate. Lactone estimations were made at 30-min. intervals for 2 hr. The following treatments were given to fresh crude extracts: ●, 50°, 25 min.; ×, 55°, 5 min.; ○, 55°, 15 min.; △, untreated and 50°, 15 min.; ▲, boiled enzyme or with no substrate.

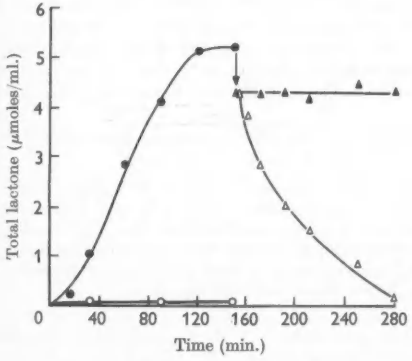


Fig. 4. Intermediate nature of the lactone synthesized by heat-treated extracts. Three identical tubes were set up with incubation mixtures as in Fig. 3. Control tube contained boiled enzyme. Lactone synthesis was followed for 2 hr. then all three tubes were plunged in a boiling-water bath to stop the reaction. After re-equilibration to 30°, to one expt. tube was added 0·5 ml. of crude extract; to the other, 0·5 ml. of boiled crude extract, and the disappearance of lactone followed at 30-min. intervals. ●, Synthesis of lactone by heat-treated extracts (mean of 2 tubes); ○, by boiled extract. △, Disappearance of accumulated lactone by the action of crude extract; ▲, by action of boiled crude extract.

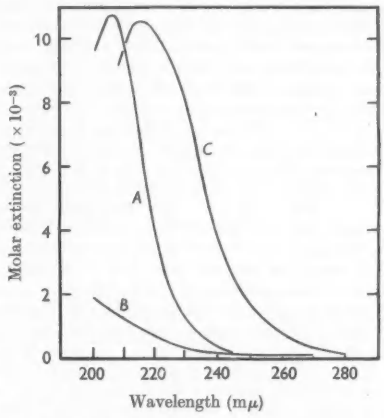


Fig. 5. Extinction curves for (±)-muconolactone (*A*), the synthetic dilactone (*B*) and β-carboxymuconolactone (*C*). All the lactones were measured as freshly prepared aqueous solutions.

Isolation and identification of the lactone

The above results with the heat-treated extracts depended upon the fortunate circumstance that both protocatechuic acid oxidase and the lactonizing enzyme were heat-stable. To conserve limited supplies of the natural substrate, β-carboxymuconic acid, protocatechuic acid was used as starting material in the preliminary experiments and in the isolation procedures. Because the oxidase requires molecular O_2 as reactant, incubations were vigorously aerated in order that the first stage in the sequence: protocatechuate → β-carboxymuconate → lactone, should not be rate-limiting.

A 1-l., three-necked flask was fitted with an electric stirrer and a narrow-bore tube, through which a rapid air-stream was blown by a pump. The flask, containing 10 m-moles of sodium protocatechuate in 500 ml. of 0·1 M-phosphate buffer, pH 7·2, was incubated in a constant-temperature water bath at 30°. A portion (100 ml.) of heat-treated extract (about 1 g. of protein) was added in two separate lots, the first at zero time, the second about 3 or 4 hr. later. The solution was kept saturated with air by agitation with the stirrer and the air stream. Frothing was minimized by the addition of 0·1 ml. of a 0·05% (w/v) solution of Silicone M.S. antifoam emulsion 'B' (Hopkin and Williams Ltd.), at about hourly intervals. A total of 0·7 ml. was added during the course of the experiment. Lactone synthesis was followed on 1 ml. samples throughout the course of the reaction, and, at completion, about 8 hr., the flask contents were brought to pH 2·5 with dil. HCl. The precipitated protein was removed by centrifuging and the supernatant solution, in two portions, extracted twice with 100 ml. of pure ether to remove any residual protocatechuic acid. In the first attempts, the aqueous solution was freeze-dried to about 60 ml., but in later

preparations the volume was decreased by evaporation under reduced pressure with the water-bath temperature not exceeding 40°. The lactone solution was then continuously extracted with pure ether for 48–60 hr., or until the hydroxamate test on the aqueous solution was minimal. Evaporation of the ether extract *in vacuo* left a pale-yellow syrup, which was difficult to crystallize. Crystallization was finally achieved by leaving the syrup in a refrigerator for some hours and scratching the side of the vessel with a glass rod. Subsequent crystallizations were more readily effected by seeding with a crystal of the isolated material. The product (250 mg.) was recrystallized twice from ethyl acetate–benzene as a white crystalline powder, m.p. 74–75°. [Sistrom & Stanier (1954) gave 76–77·5° for the (+)-isomer of muconolactone.] Further less-pure material was obtained from the residual mother liquors. The sample of synthetic (racemic) muconolactone from Professor Evans melted at 109°, and a sample of the (−)-isomer, prepared from it, at 79°. An equimolar mixture of the (−)-isomer and the biologically prepared lactone melted at 108° and did not depress the m.p. of synthetic muconolactone on admixture. The neutralization equivalent of the isolated lactone determined with an automatic titrator (Radiometer, Copenhagen, Denmark) was 142; $C_6H_6O_4$ (monobasic) requires 142. [Found: C, 50·5; H, 4·1; O (direct), 44·7. Calc. for $C_6H_6O_4$: C, 50·7; H, 4·2; O, 45·1%]. The isolated lactone was optically active and gave a value $[\alpha]_D^{20}$ +48·5±2° (mean of 10 separate determinations) in water (*c*, 4·36). The unnatural (−)-isomer gave $[\alpha]_D^{20}$ −50·5 ±2° in water (*c*, 4·40). These values are essentially the same as those recorded for muconolactone by Sistrom & Stanier (1954). The isolated lactone ran with synthetic muconolactone in solvents *C* and *D* when chromatographed; equal concentrations of natural and synthetic lactone gave equal colour intensities in the hydroxamate test and synthetic, unnatural and isolated lactones all had identical u.v. spectra ($\epsilon_{max.}$ at 207 mμ). There is little doubt therefore that the isolated lactone is (+)-muconolactone.

Conversion of protocatechuic acid and β-carboxymuconic acid into muconolactone

The degradation of either protocatechuic acid or β-carboxymuconic acid to (+)-muconolactone involves the loss of one carbon atom. The identification of (+)-muconolactone as an intermediate in protocatechuate degradation was thus surprising, since it had previously been found only as a product of catechol metabolism (Evans, Smith, Linstead & Elvidge, 1951; Sistrom & Stanier, 1954) and it was not attacked by extracts of either fully induced *Neurospora crassa* or *Pseudomonas* (Gross *et al.* 1956). The possibility remained that the lactone produced by the vibrio was indeed β-carboxymuconolactone and that decarboxylation occurred during isolation. The following evidence indicated that this was unlikely.

First, the product of the action of heat-treated extracts upon protocatechuic acid or β-carboxymuconic acid, without any manipulations, had the same spectrum as muconolactone and ran as this compound on chromatograms. β-Carboxymuconolactone is readily distinguished by both these methods. Secondly, the results of manometric experiments showed that the conversion of either protocatechuic or β-carboxymuconic acid into (+)-muconolactone by heat-treated extracts involved the evolution of CO_2 which accounted for the carbon atom which was lost. The loss of CO_2 is thus enzymic and not the result of isolation procedure. Table 3 and Fig. 6 show the metabolism of these related substrates by heated extracts of induced cells to (+)-muconolactone, and their

Table 3. *Conversion of protocatechuic acid, β-carboxymuconic acid and β-carboxymuconolactone into (+)-muconolactone and β-oxoadipic acid*

Each Warburg flask contained: phosphate buffer, 150 μmoles, pH 7·0; substrates as shown; 2 N-H_2SO_4 in side arm, or 20% KOH in centre well, 0·2 ml.; enzyme, 0·2–0·5 ml. (1·37 mg. of protein in Expt. 1; 2·5 mg. of protein in Expt. 2; 3·4 mg. of protein in Expt. 3; 3·0 mg. of protein in Expts. 4 and 5); water to a total volume of 3·0 ml. Temp., 30°. Gas phase was air when protocatechuic acid was substrate; in all other cases, incubation was performed under O_2-free N_2. (+)-Muconolactone was determined from samples of the flask contents at completion of experiments, i.e. when no further change in gas volumes occurred. Values are given as μmoles.

Expt. no.	Enzyme	Substrate	Substrate used	O_2 uptake	CO_2 evolved	β-Oxo-adipate	(+)-Muco-nolactone
1	Heat-treated	β-Carboxymuconic acid	2·9	—	2·9	0	2·3
		β-Carboxymuconic acid	3·6	—	3·2	0·2	2·3
		Protocatechuic acid	3·0	2·9	3·0	0·1	2·7
2	Crude	β-Carboxymuconic acid	4·8	—	4·6	4·7	0
		β-Carboxymuconic acid	8·0	—	7·5	7·4	0
		β-Carboxymuconic acid	10·3	—	10·2	9·6	0
3	Heat-treated	β-Carboxymuconic acid	4·8	—	4·6	0·3	4·0
		β-Carboxymuconic acid	8·0	—	7·7	0·6	7·4
		Protocatechuic acid	5·0	4·9	4·9	0	4·3
		Protocatechuic acid	8·0	7·8	8·0	—	7·4
4	Heat-treated	β-Carboxymuconolactone	3·0	—	0·7	0	—
		β-Carboxymuconolactone	5·0	—	0·3	0	—
		β-Carboxymuconolactone	8·0	—	0·2	0	—
5	Crude	β-Carboxymuconolactone	3·0	—	0·1	0	—
		β-Carboxymuconolactone	5·0	—	0·3	—	—

conversion into β-oxoadipic acid if the heat-treatment is omitted. Thirdly, the absence of any activity towards biologically prepared β-carboxymuconolactone by heat-treated or crude extracts of the vibrio grown on *p*-hydroxybenzoate (Fig. 7) confirmed the belief that this lactone was not an intermediate in protocatechuic acid degradation by this organism. Manometric experiments confirmed the absence of significant CO_2 evolution from β-carboxymuconolactone by heat-treated extracts, and also the failure of crude extracts to form β-oxoadipic acid from this substrate (Table 3).

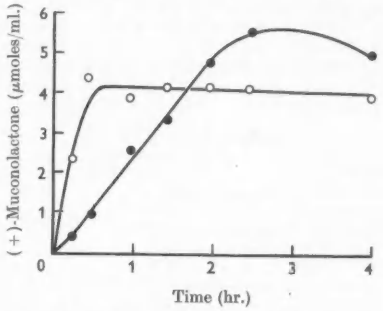


Fig. 6. Synthesis of (+)-muconolactone from some precursors by heat-treated extracts. The reaction mixtures contained: phosphate buffer, 800 μmoles, pH 7·0; substrate, 75 μmoles; enzyme, 1·0 ml. (2·65 mg. of protein); total vol., 10 ml. Temp., 30°. Incubations were performed under conditions identical with those described in Table 4. Control tubes contained boiled enzyme or no substrate. Samples were estimated for lactone at 15 and 30 min., and then at 30-min. intervals. ●, Protocatechuic acid; ○, β-carboxymuconic acid as substrates.

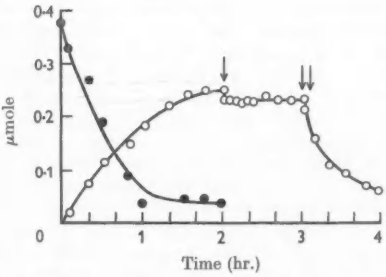


Fig. 7. Synthesis of β-carboxymuconolactone by the resolved lactonizing enzyme of *Neurospora crassa* and the lack of activity towards this substrate shown by *Vibrio* extracts. Cuvettes originally contained: tris–HCl buffer, 100 μmoles, pH 7·4; β-carboxymuconic acid, 0·4 μmole; resolved *Neurospora* lactonizing enzyme, 0·05 ml. Synthesis of β-carboxymuconolactone and disappearance of the acid were followed by extinction measurements at 225 and 270 mμ. At the completion of this reaction, 0·02 ml. (0·15 mg. of protein) of crude *Vibrio* extract was added (↓), and the extinction at 225 mμ followed for a further 1 hr. At this time, 0·05 ml. (0·15 mg. of protein) of crude *Neurospora* extract (containing the specific delactonizing enzyme) was added (↓↓), and disappearance of the accumulated lactone followed. ●, β-Carboxymuconic acid; ○, β-carboxymuconolactone. Temp., 25°.

After it was established that CO_2 was lost in conversion of β-carboxymuconic acid into (+)-muconolactone and that β-carboxymuconolactone was not an intermediate, the six-carbon analogue *cis–cis*-muconic acid was investigated as a likely intermediate. It was considered possible that active β-carboxymuconic acid was decarboxylated first and the resulting *cis–cis*-muconic acid lactonized to (+)-muconolactone; [Gross *et al.* (1956) and McDonald *et al.* (1954) discuss the stereoconfiguration of these compounds.] Crude extracts, however, did not attack *cis–cis*-muconic acid nor did heat-treated extracts synthesize any lactone from this substrate.

Lactonizing activity in unadapted cells

Protocatechuic acid oxidase is a typical adaptive enzyme formed only in response to its specific substrate or a metabolic precursor (Sleeper & Stanier, 1950; Stanier, 1950; Gross *et al.* 1956; Cartwright & Cain, 1959; Ribbons & Evans, 1960). Only in the 'aromaticless mutant' Y7655a of *Neurospora crassa* is the enzyme constitutive, and even here it might be more accurately described as auto-induced. In the vibrio, there was no attack on protocatechuic acid by extracts of cells grown with succinate as the carbon source or in peptone media, as measured by β-oxoadipate production from the substrate. Extracts from cultures of unadapted cells inoculated from slopes containing aromatic substrates often give some activity towards protocatechuic acid (0·5–2·0% of that of fully induced cultures), but, in the present experiments, large-scale cultures of unadapted cells were inoculated from a nutrient-agar slope derived by serial subculture from the original N.C.I.B. dried culture; the cells produced had thus never been in contact with an aromatic substrate. Under these conditions, there was complete absence of protocatechuic acid oxidase and with protocatechuic acid as substrate, no lactone was formed by heat-treated non-induced extracts.

Gross *et al.* (1956) detected lactonizing and delactonizing enzymes of *Neurospora crassa* in non-induced cultures of the mould. The lactonizing enzyme of *Vibrio* O1 was also present in extracts from cells grown with succinic acid but the amount of enzyme was radically reduced. By using large amounts of extract, however, with longer incubation times, measurable amounts of lactone were produced (Table 4).

Table 4. *Lactonizing activity in induced and non-induced cells*

Induced cells were grown with *p*-hydroxybenzoic acid as carbon source from inocula grown on the same medium. Non-induced cells were grown with either succinic acid or peptone as carbon source from inocula derived from the original N.C.I.B. dried culture by serial subculture on nutrient-agar slopes; such cells had never been in contact with the aromatic carbon source. Extracts of both types of cells were made with a Hughes press, and the extracts heat-treated. Incubation mixtures (in 6 in. boiling tubes) contained: phosphate buffer, 700 μ-moles, pH 7·0; β-carboxymuconic acid (active isomer) or protocatechuic acid, 50 μmoles; heat-treated extract, 1·0 ml. (16·4 mg. of protein for succinate-grown cells; 15·5 mg. of protein for peptone-grown cells, 2·7 mg. of protein for *p*-hydroxybenzoate-grown cells); total volume, 10 ml. Control tubes contained boiled enzyme. The boiling tubes were incubated in a water bath at 30°, and were vigorously shaken on a reciprocating device at 280 strokes/min. with a 5 cm. throw. Samples were estimated for lactone every 15 min. for induced extracts, every hour for non-induced extracts and the rate of synthesis was calculated from the linear response found.

Carbon source for cells	Substrate	(+)-Muconolactone formed (μmoles/hr./mg. of protein)
Succinic acid	Protocatechuic acid	0
Succinic acid	β-Carboxymuconic acid	0·04
Peptone	β-Carboxymuconic acid	0·05
p-Hydroxybenzoic acid	Protocatechuic acid	1·00
p-Hydroxybenzoic acid	β-Carboxymuconic acid	3·25

Table 5. *Stimulation of lactonizing activity in heat-treated extracts by some bivalent metal ions*

Lactonizing enzyme was assayed by decrease in extinction at 260 mμ due to disappearance of β-carboxymuconic acid (see Experimental section for details). Cuvettes contained 0·06 mg. of enzyme protein. Metal ions were added to a final concentration of 1 mM in form of the chloride or sulphate. The decrement in extinction was followed for 5 min. and calculated per min. over the initial part of the curve.

Salt added	ΔE/min.	Stimulation (+) or inhibition (−) (% of control)
None	0·072	.
$CoCl_2$	0·060	−17
$NiCl_2$	0·044	−39
$MnSO_4$	0·120	+67
$ZnSO_4$	0·030	−54
$MgSO_4$	0·150	+108
$CaCl_2$	0·120	+67
$FeSO_4$*	0·130	+82
$HgCl_2$	0	−100
None†	0·110	.
$FeCl_3$	0·060	−45

* $Fe(OH)_2$ precipitation occurred at pH 7·4; this may be an unreliable result.
† Separate experiment with fresh enzyme.

Table 6. *Effect of some metal-chelating agents on the lactonizing activity of heat-treated extracts*

Assay and other conditions were as for Table 5. Inhibitors, and metal ions, were added to a concn. of 1 mM.

Additions	ΔE/min.	Stimulation (+) or inhibition (−) (% of control)
NaN_3	0·046	−58
1:10-Phenanthroline*	0·080	−27
EDTA†	0·124	+13
EDTA*	0·076	−31
EDTA* + $MnSO_4$	0·193	+75
None	0·110	.

* Incubated with enzyme for 20 min. before addition of substrate. † Added after substrate.

Some properties of the lactonizing enzyme

Experiments with both the *cis–cis*-muconic acid-lactonizing enzyme of *Pseudomonas* (Sistrom & Stanier, 1954) and the 'decarboxylase complex' of *Nocardia* (Cain & Cartwright, 1960) demonstrated a metal requirement; for *Nocardia* this requirement was believed to be associated with a lactonizing reaction, for which tentative evidence was produced. In the present study, a similar metal requirement has been shown in the vibrio to be associated with the lactonizing enzyme converting β-carboxymuconic acid into (+)-muconolactone. Table 5 demonstrates the stimulation of the activity of heat-treated extracts by some bivalent metal ions and inhibition by heavy-metal ions (Ni^{2+}, Co^{2+}, Zn^{2+} and Hg^{2+}). The metal requirement was emphasized by the complete reversal of EDTA inhibition by Mn^{2+} ions (Table 6). The lactonizing activity was also associated with sulphydryl groups as indicated by the inhibition due to *p*-chloromercuribenzoic acid and sodium arsenate and the partial reversal of the former with cysteine. Cysteine and cysteine + Mn^{2+} ions markedly stimulated the enzyme (Table 7); hydroxylamine was strongly inhibitory. The lactonizing enzyme had a pH optimum at 8·0; phosphate buffer appeared inhibitory (Fig. 8). The enzymic activity in these experiments was determined by following the disappearance of substrate. In all cases, except the determination of a pH optimum, such results have been checked by measurement of lactone production in confirmatory experiments; this ensured that the properties described were indeed associated with the lactonizing stage.

The lactonizing enzyme of the vibrio in impure extracts, prepared with the Hughes press, showed remarkable heat-stability; only at temperatures

above 70° did the activity decrease sharply. Protocatechuic acid oxidase had a similar though less-pronounced property, its activity falling off rapidly above 55° (Fig. 9).

Equilibrium between β-carboxymuconic acid and (+)-muconolactone

At pH 7·0, the conversion of β-carboxymuconic acid into the (+)-lactone was essentially irreversible

Table 7. *Effect of some inhibitors and sulphydryl reagents on the lactonizing activity in heat-treated* Vibrio *extracts*

Conditions were as for Tables 5 and 6. Expt. 1, 0·06 mg. of enzyme protein; Expt. 2, 0·08 mg. of protein; Expt. 3, 0·4 mg. of protein, dialysed for 24 hr. against several changes of distilled water. Each experiment was performed with fresh extract.

Expt. no.	Inhibitor	Concn. (mM)	ΔE/min.	Stimulation (+) or inhibition (−) (% of control)
1	None	.	0·110	.
	NaF	5	0·056	−49
	Hydroxylamine	1	0·020	−82
	p-Chloromercuribenzoic acid	0·1	0	−100
	p-Chloromercuribenzoic acid	0·01	0·005	−95
	p-Chloromercuribenzoic acid + cysteine	0·01 2	0·053	−52
	Na_2AsO_4	1	0·039	−65
2	None	.	0·136	.
	$KAsO_2$	1	0·061	−55
	Cysteine	10	0·176	+29
	Sodium thioglycollate	1	0·114	−9
3	None	.	0·480	.
	Cysteine	1	0·530	+12
	Cysteine + $MnSO_4$	1 1	0·750	+56

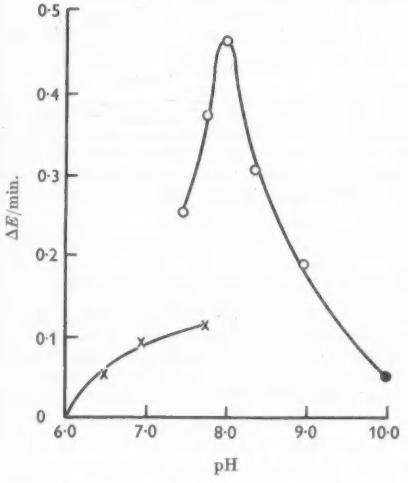


Fig. 8. pH optimum for the lactonizing enzyme in heat-treated extracts. Conditions of assay were as described in Table 5; 0·06 mg. of enzyme protein was used per cuvette. ×, Phosphate buffer; ○, tris–HCl buffer; ●, KCl–borate buffer. The pH was determined at the end of the reaction with a glass electrode.

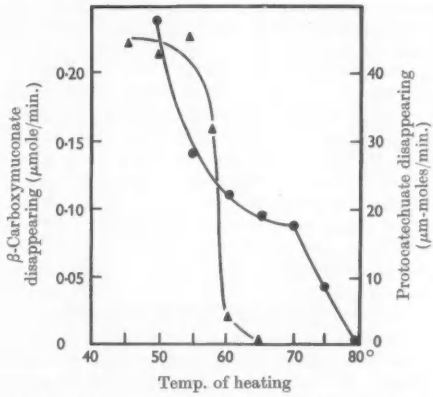


Fig. 9. Heat-stability of protocatechuic acid oxidase and the lactonizing enzyme in *Vibrio* extracts. The extracts were rapidly heated in tubes to the given temperature in a water bath, held at that temperature for 5 min., and quickly cooled by placing them in melting ice where they were held till assayed. The assay mixture per cuvette contained: tris–HCl buffer, 200 μmoles, pH 7·4; substrate, 0·5 μmole; cell extract, 0·1 ml. (1·5 mg. of protein). Assay temp., 30°. The disappearance of substrate/min. was calculated from the initial portion of the response–time curve for each batch of enzyme. ●, Lactonizing enzyme; ▲, protocatechuic acid oxidase.

in favour of lactone synthesis. This was confirmed spectrophotometrically, for the amounts of acid in mixtures of acid and lactone can be calculated from extinction measurements at 260 mμ where absorption due to the lactone is negligible. The ratio of acid to lactone at equilibrium is markedly affected by pH, since H^+ is a reactant:

$$\beta\text{-Carboxymuconate}^{3-} + 2H^+ \rightleftharpoons \text{lactonate}^- + CO_2.$$

The ratios of lactone : acid at equilibrium decreased rapidly with increasing pH, probably as a result of changes in the reacting species (Table 8).

Delactonizing activity of Vibrio O1

The delactonizing enzyme occurred at its highest levels in cells entering the stationary phase, and was strictly adaptive; no activity was found in bacteria grown with succinate as carbon source. This enzyme catalysed the essentially irreversible hydrolysis of (+)-muconolactone to β-oxoadipic acid. The synthetic lactone was only half-utilized, the (−)-isomer was not attacked, nor did it inhibit utilization of the (+)-isomer at equimolar concentrations (Table 9). The enzyme did not attack the dilactone. The delactonizing enzyme in crude extracts was much less heat-stable than protocatechuic acid oxidase or the lactonizing enzyme; it lost 85 % of its activity after 5 min. at 55°, and was completely inactivated after 15 min. at this temperature (Fig. 10). The delactonizing activity of crude extracts was slightly stimulated by several metal ions, Ca^{2+} ions giving the best stimulation, 20 %. Inhibition of delactonizing activity by 30–40 % has consistently been found with added 10 mM-EDTA, such inhibition being reversed by Ca^{2+} ions (Table 10). Dialysis for up to 3 days against several changes of distilled water did not reduce activity nor did dialysis against 1 mM-EDTA for 24 hr. The marked inhibition by *p*-

Table 8. *Effect of* pH *on the equilibrium between β-carboxymuconic acid and* (+)*-muconolactone*

Cuvettes contained: buffer, 100 μmoles; β-carboxymuconic acid, 0·5 μmole; enzyme (heat-treated extract), 0·1 mg. of protein; water to a final volume of 3 ml. Extinction decrease at each pH was followed at 260 mμ until it attained a steady value from which the amount of acid remaining was calculated. (+)-Muconolactone does not absorb at this wavelength. Allowance was made in the calculations for the 7 % inactive isomer in the purest sample of β-carboxymuconic acid available.

Buffer	pH	Acid remaining (%)	Ratio: (+)-lactone / acid at equilibrium
Phosphate	6·0	0	.
Phosphate	7·0	0·1	99·9
Tris	7·4	2·9	33·5
Tris	8·0	12·7	6·9
Tris	8·4	13·8	6·2
Tris	9·0	14·7	5·8

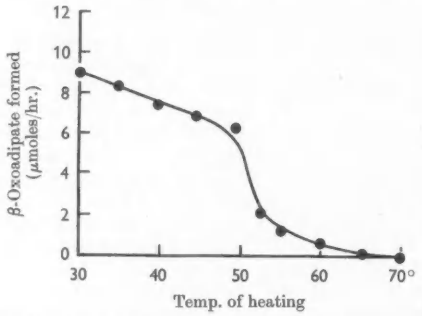


Fig. 10. Heat-stability of the delactonizing enzyme. The heat-treatment of the enzyme extracts was identical with that in Fig. 9. The assay mixture for the delactonizing activity is described in the Experimental section; flasks contained 2·25 mg. of protein. Incubation time, 20 min.; temp., 30°.

Table 9. *Conversion of the isomeric muconolactones and dilactone into β-oxoadipic acid*

Extract was derived from 48 hr.-old cells. Each Warburg flask contained: phosphate buffer, 200 μmoles, pH 7·0; substrate, 3, 5 or 6 μmoles; water to a final volume of 3 ml.; enzyme, 3·0 mg. of protein (as crude extract). Incubation time, 3 hr. β-Oxoadipic acid was determined by catalytic decarboxylation. Results are corrected for endogenous activity.

Substrate	Substrate added (μmoles)	β-Oxoadipic acid produced (μmoles)	β-Oxoadipic acid produced (μmoles/mole of substrate)
(+)-Muconolactone	3·0	3·30	1·10
(+)-Muconolactone	5·0	4·70	0·94
(+)-Muconolactone	6·0	5·70	0·95
(±)-Muconolactone	3·0	1·45	0·48
(±)-Muconolactone	5·0	2·55	0·51
(−)-Muconolactone	5·0	0	0
(+)-Muconolactone + (−)-muconolactone	5·0 + 5·0	4·90	0·98
Dilactone (synthetic)	5·0	0·30	0·06

Table 10. *Effect of some inhibitors on the delactonizing enzyme*

The assay system was that described in the Experimental section. Each flask contained 2·5 mg. of enzyme protein. After a preliminary experiment had established a linear disappearance of lactone with time, the incubation was carried out for 30 min., and the β-oxoadipic acid produced was determined by catalytic decarboxylation.

	Inhibitor	Concn. (mM)	β-Oxoadipic acid formed (μmoles/hr.)	Inhibition (%)
Expt. 1	None	.	10·8	.
	EDTA	2·0	7·4	31
	NaN_3	2·0	10·8	0
	NaF	2·0	11·4	0
	Na_3AsO_4	1·0	11·0	0
	$KAsO_2$	1·0	10·8	0
	p-Chloromercuribenzoic acid	0·1	7·0	36
	p-Chloromercuribenzoic acid + cysteine	0·01 + 2·0	9·8	9
	EDTA + $CaCl_2$	2·0 + 1·0	10·6	2
Exp. 2	None	.	6·0	.
	EDTA	10	3·4	43
	EDTA + $CaCl_2$	10 + 5	5·4	10
	p-Chloromercuribenzoic acid	0·5	1·0	84

chloromercuribenzoate was again partly reversed by cysteine. The pH optimum was about 8·4.

A partial purification of the enzyme was effected as follows. The fresh, crude, Hughes-press extract was fractionated with saturated $(NH_4)_2SO_4$ and the 0–0·45 saturated fraction collected. This fraction was taken up in water and dialysed. It was then treated with 0·05 vol. of 1·0M-$MnCl_2$, and the precipitate discarded. The supernatant was then refractionated with $(NH_4)_2SO_4$ and the fraction precipitating between 0·4 and complete saturation collected. After dialysis to remove $(NH_4)_2SO_4$, this fraction was used as source of delactonizing enzyme. This procedure effected a fourfold purification based on specific activity.

A plot of increasing substrate concentrations (S) against the reaction velocity (V), with the partially purified enzyme, gave a K_m value of 6·2 mM. A plot of S/V against V and calculating K_m from both intercepts (Lineweaver & Burk, 1934) gave a mean K_m of 10·2 mM with a $V_{max.}$ of 30 μmoles/hr. Both these values of K_m are much higher than the figure of 78 μM given for the *Pseudomonas* system by Sistrom & Stanier (1954). Inhibition of the enzyme occurred at substrate concentrations above 30 mM. The presence of Ca^{2+} ions caused a pronounced stimulation (20–30 %) of activity with the partially purified enzyme.

DISCUSSION

Pathways previously described for carbocyclic ring-fission by micro-organisms have involved either the protocatechuic acid or the catechol sequences (Stanier, 1948; Stanier, Sleeper, Tsuchida & McDonald, 1950; Sistrom & Stanier, 1953; Evans *et al.* 1951). These pathways are quite distinct up to the stage of the product, β-oxoadipic acid, from whence they enter terminal oxidation mechanisms (Kilby, 1951; Katagiri & Hayaishi, 1957; Ottey & Tatum, 1957). Gross *et al.* (1956) have shown that in *Neurospora* β-carboxymuconic acid is converted into the corresponding β-carboxymuconolactone. This mould did not attack muconolactone; direct decarboxylation of β-carboxymuconolactone does not therefore occur in this organism. On the other hand neither extracts of *Pseudomonas* grown with *p*-hydroxybenzoic acid nor those from *Nocardia* grown with *p*-nitrobenzoic acid (both of which are simultaneously adapted to metabolize protocatechuic acid) attack β-carboxymuconolactone (Gross *et al.* 1956; Cain & Cartwright, 1960). This result and those obtained with [^{14}C]-protocatechuic acid (Gross *et al.* 1956; Tatum & Gross, 1956) show that β-carboxymuconolactone is not involved in β-carboxymuconic acid degradation in these two bacteria. Results from the present study imply a similar situation in *Vibrio* O1. The vibrio differs from *Pseudomonas*, however, in that extracts of fully induced cells produce (+)-muconolactone and carbon dioxide from β-carboxymuconic acid and rapidly and completely degrade this lactone to β-oxoadipate. Decarboxylation occurs at the β-carboxymuconic acid level, for fully induced extracts do not lactonize, nor degrade further, *cis–cis*-muconic acid, which was the sole precursor of muconolactone previously known. In addition, whereas the delactonizing

enzyme of *Vibrio* O1 is strictly adaptive, both *Neurospora* and the pseudomonad which oxidizes phthalic acid, possess a delactonizing enzyme when grown with glucose as sole carbon source (Gross *et al.* 1956; Dr D. W. Ribbons, personal communication). The adaptive nature of this specific enzyme further emphasizes the true intermediary position of muconolactone on the protocatechuic acid-degradation pathway of *Vibrio* O1. In contrast with the strictly adaptive development of the delactonizing enzyme, the lactonizing enzyme of the vibrio is detectable in cells grown with succinic acid or in peptone media; nevertheless, growth in the presence of *p*-hydroxybenzoic acid causes a 75-fold increase in the enzyme.

The protocatechuic acid oxidases from *Vibrio* (this study), *Pseudomonas* (Stanier & Ingraham, 1954), *Nocardia erythropolis* (Cain & Cartwright, 1960) and the phthalate-oxidizing pseudomonad (Ribbons & Evans, 1960) are little affected by dialysis and show no requirement for Fe^{2+} ions. These contrast with the findings in *Neurospora crassa* (Ottey & Tatum, 1957) and in another *Pseudomonas* (Dagley & Patel, 1957), although the enzyme in the latter organism differs from all the others in that β-carboxymuconic acid is not the oxidation product. At the other end of the scale even crude extracts of some soil bacteria, recently examined in this laboratory, require supplementation with Fe^{2+} ions before protocatechuic acid was oxidized at an appreciable rate. If it is assumed that Fe^{2+} ions are involved in all protocatechuic acid oxidases (Mason, 1957), then in some bacteria the iron must be very tightly bound to the apoenzyme, whereas in others it is equally labile.

The properties of the lactonizing enzyme of *Vibrio* are very similar to those described for the analogous enzyme of *Pseudomonas* which uses *cis–cis*-muconic acid as substrate (Sistrom & Stanier, 1954) and to those recorded for the enzyme complex degrading β-carboxymuconic acid in *Nocardia* (Cain & Cartwright, 1960). Each has a slightly alkaline pH optimum, each shows a requirement for a bivalent metal, and the *Vibrio* and *Nocardia* enzymes have reactive sulphydryl groups associated with their activity. The *Vibrio* enzyme is distinguished from those of the other bacteria by its remarkable heat-stability and by the simultaneous decarboxylation–lactonizing activity. Enzyme-fractionation attempts have so far failed to separate the two processes.

Sistrom & Stanier (1954) briefly described some properties of the *Pseudomonas* delactonizing enzyme; the *Vibrio* enzyme closely resembles it in specificity. A consistent inhibition by chelating agents has been found in the *Vibrio* system, whereas no such effect was recorded for the *Pseudomonas* enzyme. The stimulation by Ca^{2+} ions and the reversal of EDTA inhibition by this ion also imply a metal requirement for the delactonizing step.

The results of this study present a pathway of aromatic breakdown involving intermediates previously thought to be on quite distinct sequences (Fig. 11). There has indeed been a tendency to regard the known mechanism of fission of the carbocyclic ring (i.e. opening between two hydroxyl groups *ortho* to each other) as ubiquitous and to assume that the ring-opened (aliphatic) products must be metabolized through either, *cis–cis*-muconic acid and its lactone on the catechol pathway, or β-carboxymuconic acid on the protocatechuic pathway. In the second pathway, some organisms form β-carboxymuconolactone; the majority of those at present known do not. Apart from the results in this paper which show that *Vibrio* O1 uses parts of both sequences, other recent work has cast some doubt on the supposed biochemical unity in this field. Dagley & Stopher (1959) have proposed a new mode of ring-fission of catechol in which 2-hydroxymuconic semialde-

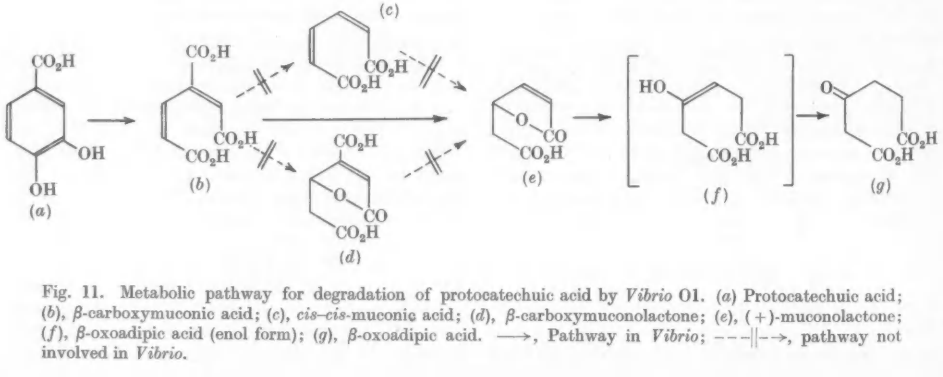
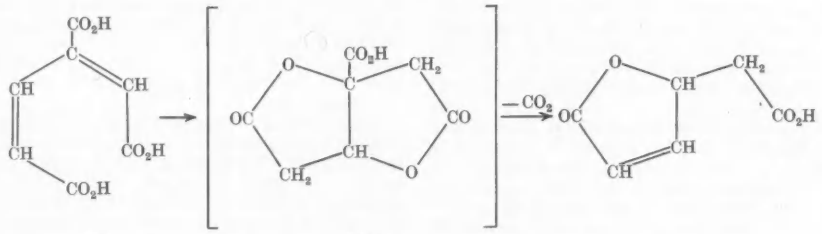


Fig. 11. Metabolic pathway for degradation of protocatechuic acid by *Vibrio* O1. (*a*) Protocatechuic acid; (*b*), β-carboxymuconic acid; (*c*), *cis–cis*-muconic acid; (*d*), β-carboxymuconolactone; (*e*), (+)-muconolactone; (*f*), β-oxoadipic acid (enol form); (*g*), β-oxoadipic acid. ⟶, Pathway in *Vibrio*; - - -||-→, pathway not involved in *Vibrio*.



(V) (IV)

Scheme I

hyde is formed instead of muconic acid. Trippett *et al.* (1960) have shown that protocatechuic acid undergoes a similar enzymic ring-cleavage to give an intermediate that reacts spontaneously with ammonia to give a pyridine derivative, 2:4-lutidinic acid, which has been isolated and characterized. The conversion of the dilactone of *cis–cis*-muconic acid into β-oxoadipic acid by extracts of the phthalate-oxidizing pseudomonad (Ribbons & Evans, 1959) offers the possibility of yet another pathway, although the observation that cells grown with glucose also perform this reaction renders its status as an intermediate in the reaction sequence uncertain at present. Nevertheless, the isotopic data of Gross *et al.* (1956) require a symmetrical intermediate between β-carboxymuconic acid and β-oxoadipic acid in the *Pseudomonas* system; the dilactone or βγ-dihydroxyadipic acid fulfil this requirement.

A further possibility arises from the present study. Although β-carboxymuconolactone is not metabolized, a double lactonization of β-carboxymuconic acid (V) would yield a carboxydilactone (IV). Decarboxylation and non-enzymic hydrolysis of the carboxydilactone (IV) to muconolactone (I) is thus possible (Scheme I).

Decarboxylation and hydrolysis in Scheme I would be required to occur simultaneously, without the appearance of the decarboxylation product [i.e. the dilactone, (II)] dissociated from the enzyme, because (II) is not attacked by the vibrio. Nevertheless (IV) would satisfy a requirement for a symmetrical intermediate in this organism should this be required to interpret isotopic data with [2-^{14}C]protocatechuate. As yet there is no evidence for it.

SUMMARY

1. Protocatechuic acid is oxidized by crude extracts of *Vibrio* O1 to β-oxoadipic acid; partially purified extracts yield stoicheiometric amounts of β-carboxymuconic acid.

2. Heat-treated extracts of the vibrio accumulate a lactone from either protocatechuic acid or β-carboxymuconic acid.

3. The lactone has been isolated and identified as (+)-4-hydroxyhex-2-enedioic 1→4-lactone [(+)-muconolactone].

4. Enzymic synthesis of (+)-muconolactone from β-carboxymuconic acid is accompanied by loss of carbon dioxide. Neither *cis–cis*-muconic acid nor β-carboxymuconolactone is attacked by vibrio extracts grown with *p*-hydroxybenzoic acid as carbon source, so the lactonization and decarboxylation appear to occur simultaneously.

5. Some properties of the lactonizing and delactonizing enzymes are described and compared with similar enzymes from other micro-organisms. In particular, a metal requirement and sulphydryl groups are associated with the lactonizing enzyme of *Vibrio*, the amount of which in cells is markedly increased by growth on *p*-hydroxybenzoic acid.

6. The significance of symmetric and asymmetric lactones is discussed in relation to new and distinct modes of aromatic ring fission.

Dr S. Dagley maintained the keenest interest throughout the duration of this work. I wish to express to him my sincerest thanks for his consistent encouragement and advice. I am indebted to Dr D. W. Ribbons and Professor W. C. Evans of Bangor for their most generous gifts of synthetic compounds, for a culture of *N. crassa* SY4a, and for making available many of their related results. Thanks are due to Professor E. L. Tatum for cultures of the *N. crassa* mutants and to Professor R. Y. Stanier and Dr D. L. McDonald, University of California, Berkeley, for a generous gift of pure β-carboxymuconic acid. The department is in receipt of a grant from D.S.I.R. towards apparatus.

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The Metabolism of Protocatechuic Acid by Certain Micro-organisms

A REASSESSMENT OF THE EVIDENCE FOR THE PARTICIPATION OF 2:6-DIOXA-3:7-DIOXO*BICYCLO*[3:3:0]OCTANE AS AN INTERMEDIATE

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A variety of microbial species are capable of the aerobic metabolism of protocatechuic acid through the β-oxoadipate pathway. Gross, Gafford & Tatum (1956), using [2:6-$^{14}C_2$]protocatechuic acid, showed that the carbonyl-C of β-oxoadipate produced by the *Neurospora crassa* enzyme system originated solely from C-6, whereas with certain *Pseudomonas* enzymes (strain, A3.12, Stanier) it was randomly derived from C-1 and C-6 of protocatechuic acid. The latter pathway clearly diverges from the former route after the *cis–cis*-β-carboxymuconic acid stage. Table 1 summarizes results showing the ability of cell-free systems of various micro-organisms, induced to protocatechuate, to form β-oxoadipate from likely precursors.

Elsden & Peel (1958) pointed out that the isotopic results of Gross *et al.* (1956) with the *Pseudomonas* system could be explained if an intermediate possessing a symmetrical structure was formed before β-oxoadipate, and suggested this might be 2:6-dioxa-3:7-dioxo*bicyclo*[3:3:0]octane (I). This dilactone was isolated by Landa & Eliasek (1956) from *Oospora* (*Geotrichum*?) cultures growing with catechol as carbon source, and had previously been synthesized by Elvidge *et al.* (1950). Ribbons & Evans (1959) repeated this synthesis, and found that both the synthetic dilactone and the compound isolated from the culture (kindly provided by Professor Landa) were converted into β-oxoadipate by crude extracts of a phthalic acid-oxidizing *Pseudomonas* sp., which also converted protocatechuate into the same product. The rate of production of β-oxoadipate from the dilactone under the conditions of this experiment, with extracts of cells grown with phthalate, was as rapid as that from protocatechuate, β-carboxymuconate or muconolactone; only the dilactone and muconolactone were rapidly attacked if the cells were grown with glucose as carbon source. This evidence appeared to support the contention that the dilactone or its open-chain counterpart, βγ-

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dihydroxyadipate, is the symmetrical intermediate preceding β-oxoadipate in the *Pseudomonas* pathway.

EXPERIMENTAL

Organisms and growth techniques. *Neurospora crassa* (SY4a) was a subculture of the original strain used by Gross *et al.* (1956) and was grown on the minimal medium described by them. Vanillic acid (0·02%, w/v) was added as an inducer of the enzymes for degrading protocatechuic acid. The phthalate-utilizing *Pseudomonas* was isolated and grown with phthalic acid as carbon source (Ribbons & Evans, 1960). *Vibrio* O1 (N.C.I.B. 8250) was grown with *p*-hydroxybenzoic acid as described by Cain (1961). *Nocardia erythropolis* was grown with *p*-nitrobenzoic acid as sole carbon and nitrogen sources (Cain, 1958). Each of these substrates induced enzymes capable of degrading protocatechuic acid in the respective organisms. *Pseudomonas fluorescens* (A3.12, Stanier) was grown as described by Sleeper, Tsuchida & Stanier (1950).

Cell-free extracts. Extracts of *Neurospora crassa* were made by grinding the mycelium with powdered glass (Cain, 1961). The preparation of extracts of the other micro-organisms has been previously described (Ribbons & Evans, 1960; Cain, 1961; Cain & Cartwright, 1960).

Chemicals. β-Carboxymuconic acid was synthesized biologically from protocatechuic acid, by using the purified protocatechuic acid oxidase from *Nocardia* (Cain & Cartwright, 1960), or the dialysed enzyme preparation from *Pseudomonas* (phthalate-utilizer) (Ribbons & Evans, 1960). β-Carboxymuconolactone was prepared as described by Gross *et al.* (1956). (+)-Muconolactone was isolated from incubation mixtures of protocatechuic acid and heat-treated *Vibrio* extracts (Cain, 1961). *cis–cis*-Muconic acid, (±)-muconolactone and the dilactone were synthesized according to the methods of Elvidge *et al.* (1950).

βγ-Dihydroxyadipic acid was synthesized by two different routes: (*a*) Ketipic ester (diethyl βγ-dioxoadipate), m.p. 76°, was prepared by the method of Wislicenus (1887) (cf. Franzen & Schmitt, 1925). The ester was reduced with aluminium amalgam and saponified with barium hydroxide, and the barium salt of βγ-dihydroxyadipic acid precipitated with ethanol according to the procedure of Pankoke (1925). (*b*) $\triangle^2$-Dihydromuconic acid (m.p. 195°) was obtained from mucic acid by the method of Ruhemann & Elliot (1890). Bromination of this, followed by hydrolysis of the product with barium hydroxide afforded the barium salt of βγ-dihydroxyadipate, isolated by precipitation with ethanol as described by Limpricht (1873).

A third route to βγ-dihydroxyadipic acid is that of Przybytek (1884) starting from erythritol via the dichlorohydrin, the oxide and the cyanhydrin, followed by hydrolysis. In our hands the yields obtained were unsatisfactory, and since Pankoke (1925) asserts that his product is similar to that of Przybytek, the latter's synthesis was not persevered with.

Estimations. β-Oxoadipate and other estimations were performed as described previously (Cain, 1961).

The rate of hydrolysis of the dilactone was estimated by two different methods, thus: (i) by following with a Unicam SP. 500 spectrophotometer the increase in E at 220 mμ due to the formation of the stronger-absorbing (±)-muconolactone; or (ii) by following the formation of the liberated carboxyl group at increasing pH values, by titrating it with 0·1 N-NaOH in an Automatic Titrator (pH-Stat) (Radiometer Ltd., Copenhagen). The Automatic Titrator consisted of an electrometric titration assembly, an automatic titrator and a recording unit. The reaction cuvette was maintained at 30° by circulating water from a constant-temperature bath with a Circotherm unit (Braun, Melsungen, W. Germany) controlled by a contact thermometer.

RESULTS AND DISCUSSION

Extracts of *Vibrio* O1 and *Nocardia erythropolis*, adapted to protocatechuate, attacked the dilactone over a 2 hr. incubation period (Table 1), but the rate of attack on the dilactone by these extracts

Table 1. *Ability of cell-free systems of various micro-organisms, induced to protocatechuate, to produce β-oxoadipate from suspected precursors*

Reaction mixtures of each system in Warburg flasks contained: phosphate buffer, pH 7·0, 100 μmoles; cell-free extract as indicated (1·0 ml. = 3 mg. of protein); substrates, 5 μmoles, in side arm, as indicated; water to a final volume of 3·0 ml. Reactions were initiated by addition of substrate, and, after incubation for 2 hr. at 30°, a sample (2 ml.) from each flask was pipetted into new Warburg flasks and brought to pH 4·0 with 1 N-acetic acid. Decarboxylation of β-oxoadipate was initiated by addition of 0·1 M-4-aminoantipyrine (0·4 ml.). Control flasks contained reaction mixtures without substrate. The values recorded, which are expressed as μmoles of β-oxoadipate formed, are corrected for the controls. Temp., 30°. ×, Not tested; −, not attacked; +, rapidly attacked. [No figures are given by Gross *et al.* (1956) for *Pseudomonas* A3.12.]

Substrate	Extracts derived from				
	Neurospora crassa SY4*a*	*Pseudomonas* A3.12 Stanier	*Pseudomonas* (phthalate-utilizer)	*Vibrio* O1	*Nocardia erythropolis*
Protocatechuic acid	4·6	+	4·9	4·8	5·0
β-Carboxymuconic acid	4·1	+	4·2	4·3	4·3
β-Carboxymuconolactone	4·9	−	0·4	0·3	0·2
(+)-Muconolactone	0·1	−	×	4·8	4·7
(±)-Muconolactone	0·2	−	2·5	2·4	2·3
cis–cis-Muconic acid	×	−	0·1	0	0·1
Dilactone (synthetic)	0·1	−	2·4	0·3	1·2
Dilactone (isolated from *Oospora*)	0·15	−	2·1	0·3	×

was very much slower than it was on protocatechuate, β-carboxymuconate or muconolactone. Extracts of unadapted cells of *Vibrio* O1 attacked neither muconolactone nor the dilactone (Cain, 1961).

The synthetic dilactone (m.p. 127°) was examined in an attempt to identify the probable lactone-like compound formed by *Nocardia* in the dissimilation of protocatechuate (Cain & Cartwright, 1960). The dilactone and muconolactone were readily distinguished in aqueous solution spectrophotometrically, since the former had no absorption peak down to 200 mμ whereas muconolactone absorbs maximally at 207 mμ (Cain, 1961); both therefore differ from the unknown compound of *Nocardia* ($E_{max.}$ at 215–218 mμ).

In their original description of the dilactone, Elvidge *et al.* (1950) merely stated that on titration at room temperature it consumed 1 equiv. of sodium hydroxide, but at 50° 2 equiv. was rapidly taken up. As no other information was given, we further investigated the hydrolysis properties at different pH values. When the dilactone was added to reaction mixtures buffered at pH 7·0 or higher, in cuvettes, an unexpected rapid increase in E at 220 mμ occurred. This change was non-enzymic, both boiled and boiled dialysed extracts giving the same result, which was finally traced to the pH of the solution. Figs. 1 and 2 show the rate of hydrolysis at various pH values measured spectrophotometrically, and by titration of the liberated carboxyl group in a pH-Stat, respectively. The hydrolysis product had the spectrum of, and showed chromatographic behaviour in solvents *C* and *D* (Cain, 1961) identical with that of an authentic sample of muconolactone; the hydrolysis product was also converted by crude extracts of *Vibrio* O1 and *Pseudomonas* (phthalate-utilizing strain) into β-oxoadipate, at a rate equivalent to that of muconolactone. The spontaneous hydrolysis of the dilactone over this pH range thus yields 4-hydroxyhex-2-enedioic 1→4-lactone (muconolactone, II). The dilactone possesses two similar asymmetric carbon atoms, and can thus exist as the (+),(−), *meso* and racemic forms. The synthetic compound (10 μmoles), after overnight hydrolysis at pH 7·8 in Warburg flasks, gave (±)-muconolactone, of which only the (+)-isomer was used by *Nocardia* or *Vibrio* extracts, as measured by the β-oxoadipate

Fig. 1. Hydrolysis of the dilactone at different pH values recorded spectrophotometrically. Phosphate (●) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl (■) buffers were used. Hydrolysis rate was measured by extinction increment at 220 mμ [ε (dilactone) = 840; ε (muconolactone) = 4670] calculated per hour from the initial portion of the time–increment curves. Each cuvette contained: buffer, 200 μmoles; dilactone, 0·5 μmole; water to a final volume of 3 ml. Temp., 22°.

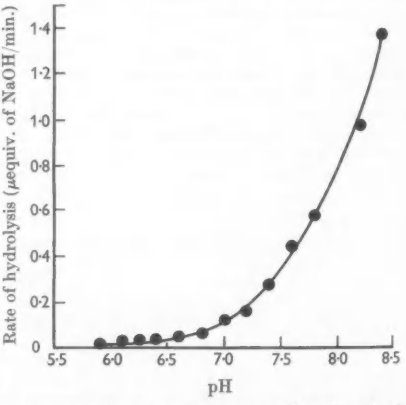
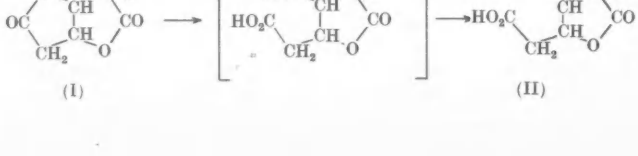


Fig. 2. Hydrolysis of the dilactone at different pH values recorded titrimetrically. The titration cup contained 4·5 ml. of a solution of the dilactone (20 μmoles/ml.). Hydrolysis at each pH was recorded graphically by the automatic addition of 0·1 N-NaOH. Temp., 30°.



(I) (II)

(5 μmoles) produced. The dilactone sample, originally isolated from catechol cultures by Landa & Eliasek (1956) after similar hydrolysis, was also only half-utilized. Moreover, the identity of the melting points of both synthetic and isolated materials (127°), which were undepressed on admixture of the materials, strongly supports the original view of Landa & Eliasek (1956), that their material was an artifact probably derived during isolation from muconolactone or *cis–cis*-muconic acid produced from catechol in their cultures.

Fig. 3 shows the relative rates of β-oxoadipate formation by extracts of *Pseudomonas* (phthalate-utilizing strain) at pH 6·25, acting on protocatechuate, muconolactone and the dilactone; at this pH, the rate of non-enzymic hydrolysis of the dilactone is low. It is apparent from this result that the spontaneous hydrolysis of the dilactone to muconolactone at higher pH values, and enzymic attack on the latter, are responsible for suspected attack on the dilactone itself, and the contention that the dilactone is an intermediate cannot be true. A similar result has since been obtained with *Nocardia* extracts at pH 6·6.

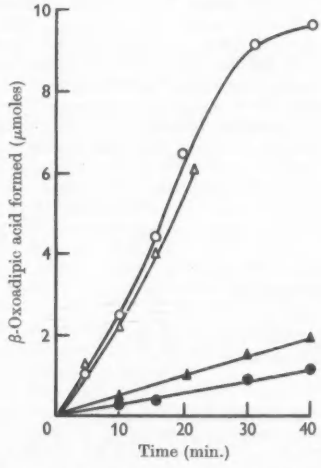


Fig. 3. Relative rates of β-oxoadipate formation by cell-free extracts of *Pseudomonas* sp. (phthalate-utilizing strain). Each Warburg flask contained: cell-free extract, 1 ml.; phosphate buffer, pH 6·25, 100 μmoles; water and substrate as indicated to a final volume of 3·0 ml. The amount of substrate was 10 μmoles (calculated for racemic mixtures of the dilactone and muconolactone was 20 μmoles). Atmosphere, air; temp., 30°. β-Oxoadipate was estimated by catalytic decarboxylation with 4-aminoantipyrine. One sample (2 ml.) from each flask, after various reaction times as indicated, was pipetted into a new Warburg flask containing 1 N-acetic acid (1 ml.) in the main compartment, and 0·1 M-4-aminoantipyrine (0·4 ml.) in the side arm. The β-oxoadipate formed was estimated immediately after equilibration at 30°. Substrates were: ○, protocatechuate; △, muconolactone; ●, dilactone. The rate of non-enzymic hydrolysis of the dilactone is also shown (▲).

The possibility that $\beta\gamma$-dihydroxyadipic acid is the symmetrical intermediate required from the tracer evidence of Gross *et al.* (1956) was also tested. Extracts from *P. fluorescens* A3.12 and *Pseudomonas* sp. (phthalate-utilizer) were incubated with two different preparations of this compound. No β-oxoadipate was detected after incubation with either extract.

The incubations were performed as follows. Each reductase tube contained: 0·2 M-phosphate buffer, pH 6·2, 1 ml.; cell-free extract, 1 ml. (containing about 15 mg. of protein); $\beta\gamma$-dihydroxyadipic acid, approx. 5 μmoles in 0·2 ml.; water to a final volume of 3·0 ml. Reactions were initiated by addition of substrate, and, after incubation for 20 min. at room temperature (approx. 20°), 1 ml. samples were saturated with ammonium sulphate; 2–3 drops of dilute sodium nitroprusside solution, followed by 0·5 ml. of conc. aq. ammonia soln. were added. An intense purple colour developed in 5 min. when β-oxoadipate was produced (Rothera, 1908). Control incubations, with no substrate, with protocatechuate and muconolactone as substrates, and with boiled extracts, were carried out simultaneously.

The negative results obtained with the two samples of $\beta\gamma$-dihydroxyadipic acid indicate that neither is an intermediate in the conversion of protocatechuate into β-oxoadipate by *P. fluorescens* (A3.12) or *Pseudomonas* sp. (phthalate-utilizer). $\beta\gamma$-Dihydroxyadipic acid possesses two asymmetric carbon atoms, and can therefore exist as the (+), (−), *meso* and racemic forms; the inability of these $\beta\gamma$-dihydroxyadipic acid preparations to act as precursors for β-oxoadipate in this enzyme system could be due to the samples' being biologically inactive forms of the compound. Pankoke (1925) maintained that his product was similar to that of Przybytek (1884) but different from that of Limpricht (1873); it is not known whether these correspond to the *meso* and racemic forms.

Protocatechuate degradation by extracts of *P. fluorescens* (A3.12) does not appear to proceed through the symmetrical intermediates, dilactone or $\beta\gamma$-dihydroxyadipic acid; extracts of *Pseudomonas* sp. (phthalate-utilizer) do not attack these substrates either, but do form β-oxoadipate from muconolactone; thus the pathway in this organism appears similar to that found for *Vibrio* O1 (Cain, 1961), and dissimilar to the *N. crassa* and *P. fluorescens* (A3.12) pathways. The tracer evidence of Gross *et al.* (1956) for *P. fluorescens* (A3.12) may not therefore be applicable to *Vibrio* O1 and *Pseudomonas* sp. (phthalate-utilizer).

SUMMARY

1. 2:6-Dioxa-3:7-dioxo*bicyclo*[3:3:0]octane ('the dilactone') hydrolyses spontaneously and non-enzymically to (±)-muconolactone; this hydrolysis is rapid above pH 7·0.

2. At pH 6·2–6·6, where the hydrolysis rate is low, extracts of *Pseudomonas* (phthalate-utilizer), *P. fluorescens* (A3.12) and *Nocardia* do not metabolize the dilactone at a rate in excess of its rate of hydrolysis.

3. The dilactone is now considered not to be an intermediate in protocatechuate metabolism by these micro-organisms.

4. Two samples of $\beta\gamma$-dihydroxyadipic acid failed to act as precursors of β-oxoadipate when used as substrates in the enzyme system which converts protocatechuate into β-oxoadipate.

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Studies on Phospholipids

7. THE DISTRIBUTION OF COMPLEX PHOSPHOLIPIDS IN VARIOUS SPECIES AND TISSUES*

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Collins & Wheeldon (1957) have advanced evidence for the occurrence in rat liver, ox brain and egg yolk of 'complex' forms of amino-phospholipids and Collins (1959*b*) has described their separation and characterization by means of countercurrent distribution. The nature of these 'complex' phospholipids has been discussed by Collins (1960), who has presented evidence that they contain a phosphate triester group and at least two atoms of phosphorus/molecule. This paper is concerned with the distribution of these complex phospholipids in rat tissues and in other biological material.

* Part 6: Collins (1959*a*).

EXPERIMENTAL

Materials

Rats. The livers, brains, hearts, small intestines, kidneys, lungs, spleens and testes of twenty white rats (Wistar strain, body wt. 150–250 g.) were removed, quickly frozen and stored at −18° until required. For the additional experiments on the countercurrent distribution of the phospholipids animals were killed as required and the tissue was extracted as rapidly as possible.

Hen's eggs. These were not more than 24 hr. old.

Cabbage leaf. Extraction was carried out within 3 hr. of collection.

Influenza virus and hen's-egg allantoic membrane. These were received in a frozen-dried condition and were prepared

by Mr G. L. Ada, Walter and Eliza Hall Institute, Melbourne (Ada & Perry, 1954).

Human-plasma fraction. A lipoprotein-rich fraction (1 g.) of human plasma (fraction IV–1, Cohn *et al.* 1946) was used. This was equivalent to 238 l. of plasma and was supplied, frozen-dried, by the Commonwealth Serum Laboratory, Melbourne.

Ox-spinal-cord sphingolipids. A mixture of cerebrosides and sphingomyelin was prepared as described by Carter, Haines, Ledyard & Norris (1947) and contained 0·77% of phosphorus and 11·8% of galactose.

Human brain. Samples of human-brain-lipid fractions were supplied by Dr P. Fantl, Baker Medical Research Institute, Alfred Hospital, Melbourne.

Yeast. Fresh compressed yeast was extracted and the lipids were treated as described below.

Freshwater crustacean (Cherix albidus *Clark*). The live crustaceans were brought to the laboratory, the whole animals were extracted and the lipids treated as described below.

Methods

The lipids were extracted and caused to react with 1-fluoro-2:4-dinitrobenzene as described by Wheeldon & Collins (1957). The product was treated with diazomethane and separated into a non-polar and a polar fraction by countercurrent distribution (Collins & Wheeldon, 1957). On each fraction phosphorus and amino nitrogen (*N*-2:4-dinitrophenyl groups) were determined as described by Wheeldon & Collins (1957). Ethanolamine and serine were determined on the polar and non-polar fractions of rat-tissue lipids by the method of Collins & Wheeldon (1958) and inositol, sugar and acyl ester groups were estimated as described by Collins (1959*b*).

Choline. The hydrolysis procedure described by Wheeldon & Collins (1958) when applied to lipids other than those from liver and brain yielded less choline than was obtained by more vigorous hydrolysis. As a consequence analyses reported here were carried out after hydrolysis by heating with 6N-hydrochloric acid in a sealed tube at 110° until all the phosphorus was present as inorganic phosphate. This usually required 24 hr. but with spleen, heart and lung lipids 48 hr. was required. Choline was determined on the hydrolysate as described by Wheeldon & Collins (1958), except that in the development of the colour the stannous chloride solution was added after the addition of the ethanol.

Phosphatidic acid-like materials. The difference between the total phospholipids in the non-polar fraction and the amounts of phosphatidylethanolamine and phosphatidylserine was used as a measure of the phosphatidic acid-like materials; these correspond to fractions X, Y and Z described by Collins (1959*b*).

Complex phospholipids. In the experiments described in Tables 1 and 2 the complex phospholipids were estimated from the amounts of dinitrophenyl groups in the polar fractions (Collins, 1959*b*). The results shown in Table 4 represent the amounts of complex phospholipids as isolated by countercurrent distribution (e.g. tubes 35–120 in Fig. 1*b*). Both procedures give the same values for dinitrophenyl groups in the complex phospholipids but only the second gives a direct estimate of their phosphorus content.

Countercurrent distribution of phospholipids. In the course of this work it was desired to examine certain rat-tissue phospholipids in greater detail. The dinitrophenylated and methylated lipids prepared as described above were separated into an acetone-soluble and an acetone-insoluble fraction by the procedure described by Collins & Shotlander (1960), by chromatography on cellulose at −18° with acetone. These two fractions were submitted to countercurrent distribution (120 transfers) with carbon tetrachloride–methanol–water (62:35:3·15, by vol.) for the acetone-soluble material and carbon tetrachloride–chloroform–methylene chloride–methanol–water (25:15:10:40:10, by vol.) for the acetone-insoluble material: the solvent systems are those described by Cole, Lathe & Ruthven (1953). The volume of each phase was 20 ml. and after distribution the contents of the first and every fifth tube were analysed for *N*-2:4-dinitrophenyl groups as described above and phosphorus as described by Collins (1959*b*).

It was essential that the above-mentioned solvent mixtures were prepared immediately before use and checked for neutrality. The acidity of the solvent mixtures was tested as follows: to 10 ml. each of water and of solvent mixture was added 0·1 ml. of Universal Indicator (British Drug Houses Ltd.). The carbon dioxide was removed under reduced pressure and the resulting colour in the aqueous phase visually compared with the colour produced in the absence of solvent. If the colour was the same (light green) the solvent mixture was acceptable, otherwise it was rejected.

RESULTS

Collins & Wheeldon (1957) showed that a simplified countercurrent distribution of dinitrophenylated and methylated phospholipids yielded two fractions which were designated polar and non-polar respectively. Collins (1959*b*) has shown that the non-polar fraction contains the phosphatidylethanolamine, phosphatidylserine and phosphatidic acid-like materials and that the polar fraction contains the complex phospholipids. Analysis of the non-polar and polar fractions of rat tissues are shown in Table 1 and in other species in Table 2. In addition to the above-mentioned results, countercurrent distribution has shown the presence of complex phospholipids in the lipids from human brain, yeast and a freshwater crustacean (*Cherix albidus* Clark).

The lipids from rat liver, heart and kidneys were studied in greater detail by means of countercurrent distribution. As lecithin and some of the complex phospholipids had similar partition coefficients in carbon tetrachloride–methanol–water a preliminary separation was necessary, and the chromatographic procedure described by Collins & Shotlander (1960) with cellulose and elution with acetone at −18° was used to separate the phospholipid derivatives into two fractions. The acetone-soluble fraction contained all the dinitrophenyl derivatives, some of the choline and some phosphatidic acid-like material. Choline and inositol were present in the acetone-insoluble fraction.

Table 1. *Distribution of phospholipids in rat tissues*

All figures, unless shown otherwise, are molar % of total lipid phosphorus.

	Brain	Heart	Intestine	Kidney	Liver	Lung	Spleen	Testes
Total lipid (% of fresh tissue)	10·1	2·3	4·8	3·6	3·6	4·7	2·2	2·6
Total lipid phosphorus (μmoles/g. of fresh tissue)	82	18·5	7·2	25	30·7	15·0	16·2	13·9
Non-polar fraction								
Phosphatidic acid-like material	9	7	6	1	2	3	4	3
Ethanolamine	19	17	19	20	13	9	11	16
Serine	4	3	3	6	2	2	2	4
Polar fraction								
Inositol	2	3	6	6	7	3	4	2
Ethanolamine	21	17	16	13	11	13	17	12
Serine	6	4	3	6	4	2	6	6
Choline	39	46	47	50	64	64	58	48
Sum of phospholipid components	100	97	100	102	103	96	102	91

Table 2. *Complex phospholipids in species other than the rat*

Non-polar fractions contain the phosphatidylethanolamine and phosphatidylserine; polar fractions contain the complex phospholipids.

Sample	Total lipid (% of wet wt.)	Phosphorus (μmoles/100 mg. of total lipid)	Ethanolamine + serine (μmoles) as % of total lipid-phosphorus (μg.atoms)	
			Non-polar fraction	Polar fraction
Hen's-egg yolk	34	26	7·7	18·1
Hen's-egg allantoic membrane	12·1	26	16·8	19·5
Influenza virus	38	32	23·8	27·8
Human-plasma fraction	30	24	3·3	7·1
Cabbage leaf	0·57	18	13·1	10·3

Table 3. *Details of material used for the counter-current-distribution experiments*

	Liver	Kidney	Heart
No. of animals	1	4	20
Tissue (g.)	11·5	8·6	16·5
Lipid (mg.)	544	276	447
Acetone-soluble derivatives (μg.atoms of P)	280	126	269
Acetone-insoluble derivatives (μg.atoms of P)	175	75	133

Table 3 shows the details of the rat-liver, -kidney and -heart lipids which were subjected to countercurrent distribution. The results of countercurrent distribution of rat-liver lipids are shown in Fig. 1 and five main fractions could be distinguished:

(1) In the acetone-insoluble portion the material in tubes 1–5 contained phosphorus but no ethanolamine, serine, choline or inositol and will be designated phosphatidic acid-like material.

(2) Tubes 6–40 in the acetone-insoluble fraction contained phosphorus and choline in the molar ratio of 0·98 and had 1·96 ester groups/g.atom of phosphorus, which are the values expected for lecithin.

(3) In the acetone-insoluble portion the contents of tubes 41–120 are designated minor phospholipids, as there was insufficient material for a detailed analysis. Several fractions could be distinguished and both inositol and choline were present.

(4) In the acetone-soluble portion the contents of tubes 1–20 correspond to the non-polar fraction previously described (Collins, 1959*b*).

(5) The remainder of the acetone-soluble portion (tubes 31–120) correspond to the complex phospholipid fraction, which clearly contains several components.

The phospholipid derivatives from heart and kidney gave similar distributions, which could be grouped into the same five fractions, but within these fractions some differences were apparent. Although the lecithin peak was in the same position in each case it was found to be skew for the kidney-phospholipid derivatives (see Fig. 1*a*), and this was thought to be due to sphingomyelin. To test this, spinal-cord sphingolipids were treated in the same way as rat-tissue lipids and the acetone-insoluble fraction was distributed in carbon tetrachloride–chloroform–methylene chloride–methanol–water. Most of the material (69%) was present in the region of tubes 10–50; sugar (as galactose) was maximal at tube 20 and phosphorus at tube 30. Hence it may be concluded that the bulge in the region of the lecithin peak is indicative of sphingomyelin and this fraction will be designated lecithin and sphingomyelin.

Differences were noted in the distribution of the minor phospholipids but the small amounts present make a comparison difficult at this stage. The complex phospholipid derivatives were distributed in the same region, in each case, but the profiles of the distribution curves differed.

The areas under the curves were summed for each of these five fractions both for phosphorus and for dinitrophenyl groups and the amounts present are shown in Table 3. The total phosphatidic acid-like material represents the sum of the acetone-soluble and -insoluble portions.

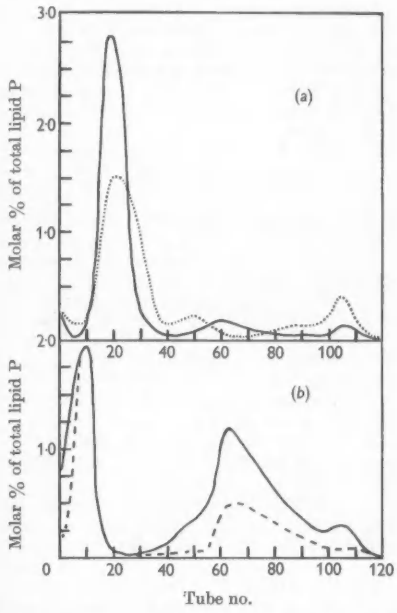

Fig. 1. Countercurrent distribution of rat-liver-phospholipid derivatives. (*a*) The acetone-insoluble fraction was distributed in carbon tetrachloride–chloroform–methylene chloride–methanol–water. (*b*) The acetone-soluble fraction was distributed in carbon tetrachloride–methanol–water. Every fifth fraction was analysed for phosphorus (——) and dinitrophenyl groups (- - - - - -). For comparison the distribution is also given for the acetone-insoluble kidney phospholipids in terms of phosphorus (.....).

DISCUSSION

The analyses for rat-tissue phospholipids are in good agreement with published data for total phospholipids and choline (Wittcoff, 1951; Dueul, 1955; Dawson, 1957; Hanahan, Dittmer & Warashina, 1957; Marinetti, Erbland & Stotz, 1958; Sakagami, Shimojo & Yokoyama, 1959). The inositol content of the rat-tissue lipids is in agreement with that found by Taylor & McKibbon (1953) for dog tissues and with Dawson (1960) for sheep liver, but is higher than that found by Marinetti *et al.* (1958) for rat liver and kidney. In recent years, with the advent of improved analytical procedures, several detailed analyses of tissue phospholipids have been published, and these results account for all or nearly all of the lipid phosphorus. By using silicic acid chromatography Hanahan *et al.* (1957) have examined rat-liver, ox-liver and yeast phospholipids and Sakagami *et al.* (1959) have studied rat-liver and -heart phospholipids. Marinetti *et al.* (1958) have analysed the phospholipids of rat liver, kidney, spleen, lung and heart by paper chromatography on silicic acid-impregnated paper. The procedure for the isolation of characteristic phosphate esters by hydrolysis of the phospholipids described by Dawson (1960) has been applied to sheep liver, and these results are included in the present comparison because of the contrast in the analytical method with those of other workers.

Except for testes the results shown in Table 1 account for all the lipid phosphorus within 4%.

Table 4. *Liver, heart and kidney phospholipids*

A comparison of the results obtained in the present investigation (A) with those obtained by other workers: [(B) Marinetti, Erbland & Stotz (1958); (C) Hanahan, Dittmer & Warashina (1957); (D) Dawson (1960)]. —, Denotes not detected. All figures are molar % of total lipid phosphorus.

	Liver				Heart			Kidney		
	(A) Rat	(B) Rat	(C) Rat	(D) Sheep	(A) Rat	(B) Rat	(D) Sheep	(A) Rat	(B) Rat	(D) Sheep
Phosphatidic acid-like material	5	3	—	3	11	10	14	7	5	7
Phosphatidylethanolamine and phosphatidylserine	17	22	26	31	19	30	29	20	30	33
Complex phospholipids	38	—	—	—	41	—	—	38	—	—
Lecithin and sphingomyelin	33	66	60	59	23	49	46	26	55	51
Inositol and other minor phospholipids	7	5	7	6	6	6	4	9	7	4

There is general agreement between these results and those of other workers that the choline, ethanolamine and serine phospholipids together account for between 85 and 95%, depending on the tissue. The remainder is represented by the inositol phospholipids and the phosphatidic acid-like material. In Table 4 it will be seen that the amounts of phosphatidic acid-like materials agree for any one tissue, notwithstanding the contrasts in the methods employed.

Table 4 shows that the major difference between the results of the present investigation and those obtained by other workers is the demonstration of the presence of complex phospholipids, which contain ethanolamine, serine and choline. This will account for the observed decrease in the proportion of phosphatidylethanolamine and phosphatidylserine and of lecithin and sphingomyelin as compared with the results of other workers. Collins (1960) has presented evidence showing that these complex phospholipids are not artifacts and has suggested that they are triester phospholipids. Evidence was also presented showing that chromatography on silicic acid could cause a breakdown of the complex phospholipids, which would account for the failure by other investigators to detect these compounds. Since tertiary phosphate esters are easily hydrolysed by 0·2N-alkali at 35° (Vernon, 1957) this would explain why the procedure described by Dawson (1960) does not distinguish between triester and diester phospholipids.

The presence of complex phospholipids has not only been demonstrated in animal tissues but also in yeast, a virus and a plant tissue. Hence it seems probable that this group of compounds is of universal occurrence.

SUMMARY

1. Analysis of the phospholipids of rat tissues agreed satisfactorily with published results except for the presence of a complex phospholipid fraction.

2. Complex phospholipids were shown to be present in rat tissues, egg yolk and allantoic membrane, influenza virus, human plasma, cabbage leaf, human brain, yeast and a freshwater crustacean.

3. The complex phospholipids constitute about 40% of all phospholipid mixtures so far examined and a procedure for isolating derivatives of these materials is described.

We wish to thank Mr G. L. Ada of the Walter and Eliza Hall Institute, Melbourne, and the Director, Commonwealth Serum Laboratories, Melbourne, for the gift of materials, and the National Health and Medical Research Council for financial support. One of us (V. L. S.) had a Commonwealth Postgraduate Award and a grant from the University of Melbourne. We wish to express our appreciation for the technical assistance of Mrs M. Labutis and Miss T. B. G. Kaak.

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Studies on Phospholipids

8. PHOSPHOLIPIDS IN RAT-LIVER MITOCHONDRIA AND MICROSOMES

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Work by Marinetti, Erbland & Stotz (1958) on pig-heart-cell fractions and by Macfarlane, Gray & Wheeldon (1960) on rat-liver-cell fractions have indicated that the mitochondrial and microsomal phospholipids may differ significantly in composition. In view of the results obtained by Collins (1960) and by Collins & Shotlander (1961), showing the widespread occurrence of complex amino-phospholipids, which have not been detected by other workers, it was important to examine the phospholipids in liver-cell fractions with the procedures described by Collins & Shotlander (1961).

EXPERIMENTAL

Preparation of the lipids of rat-liver-cell components. The livers of three male rats (Wistar strain, body wt. 200–250 g.) were treated as described by Hogeboom (1955) and a nuclear, mitochondrial, microsomal and a supernatant fraction were obtained. The lipids were extracted from these fractions and washed with acid as described by Wheeldon & Collins (1957).

Fractionation of phospholipid derivatives. The phospholipids of the mitochondria and microsomes were treated and distributed in the same way as described for whole rat-tissue lipids by Collins & Shotlander (1961). After each distribution the contents of the first and every fifth tube were diluted to 50 ml., by the addition of ethanol, and phosphorus and dinitrophenyl groups were determined.

The contents of tubes 1–35 from the distribution of the acetone-soluble fraction were combined and redistributed in *n*-heptane–methanol for 120 transfers. After this distribution the contents of the first and every fifth tube were diluted to 50 ml. with chloroform. Analyses were made for phosphorus and for dinitrophenyl groups with a value of $\epsilon_{max.}$ of 16 000 at 340 mμ.

Cardiolipin. A sample (3 mg.) of cardiolipin (obtained from Dr P. Plackett, C.S.I.R.O. Division of Animal Health, Melbourne) was washed with acid and dinitrophenylated and methylated as described above for the rat-liver lipids. The product was completely soluble in acetone, contained no dinitrophenyl groups and was distributed for 120 transfers in *n*-heptane–methanol.

Lysolecithin. A sample (3 mg.) of lysolecithin (obtained from Miss H. Doery, Commonwealth Serum Laboratories, Melbourne) was washed with acid, dinitrophenylated and methylated. The product was completely acetone-insoluble, contained no dinitrophenyl groups and was distributed for 120 transfers in carbon tetrachloride–chloroform–methylene chloride–methanol–water (25:15:10:40:10, by vol.).

Table 1. *Phospholipids of rat-liver-cell fractions*

Four rats (285–295 g. body wt.) yielded livers weighing a total of 42·7 g. Cell fractions were prepared and the lipid contents are shown below.

	Lipid (mg.)	Phosphorus (% of lipid)	Lipid (% of fresh wt. of liver)
Nuclear fraction	474	2·9	1·1
Mitochondria	286	3·5	0·7
Microsomes	529	3·2	1·2
Supernatant	138	0·8	0·3
Total	1427		3·3

Analytical procedure. The analytical procedures for phosphorus and dinitrophenyl groups are those described by Collins (1959).

Solvents. These were redistilled and care was taken that the solvent mixtures used for countercurrent distribution were neutral (Collins & Shotlander, 1961).

RESULTS

Table 1 shows the proportion of total lipid and lipid phosphorus in rat-liver-cell fractions. The lipids from the nuclear fraction were not further investigated as this cell fraction was contaminated with non-nuclear material, and there was insufficient for the study of the lipids in the supernatant fraction.

The distribution of the phospholipids from mitochondria and microsomes was similar to those obtained for whole rat-liver lipids (Collins & Shotlander, 1961) but certain differences between the two cell fractions were noted.

The distribution of the acetone-soluble portions (Fig. 1) of mitochondrial lipids shows a peak at tube 5, which is not apparent in the microsomal distribution, whereas the main peak in the complex phospholipid fraction is at tube 81 for mitochondria and at tube 70 for microsomes. The position of the maximum in the distribution of the phosphatidylethanolamine and phosphatidylserine derivatives is at tube 17 in both cases, which demonstrates that the other differences are not due to small variations in the composition of the solvent systems or to temperature effects.

The contents of tubes 1–35 were combined and redistributed in *n*-heptane–methanol (Fig. 2). The phosphatidylethanolamine and phosphatidylserine were maximal at tube 38 in both cases. The two distributions differed mainly in the amount of lipid phosphorus maximal at tube 95; there was little or no lipid phosphorus in this region in the microsome lipids but substantial amounts with the mitochondrial lipids.

When a sample of cardiolipin was dinitrophenylated and methylated and then distributed in heptane–methanol the material was maximal at tube 89. Hence it may be concluded that the material present in mitochondrial lipids, but present only in trace amounts in microsomes, is probably cardiolipin.

A comparison of the distribution of the acetone-insoluble lipids is shown in Fig. 3. Here there are differences in the minor phospholipids (tubes 40–120) but their importance is difficult to assess owing to the small amounts of material involved. In both cases the main peak, due to lecithin (Collins & Shotlander, 1961), is at the same position. After treatment with 1-fluoro-2:4-dinitrobenzene, and with diazomethane, lysolecithin was insoluble in acetone and, on distribution in carbon tetrachloride–chloroform–methylene chloride–methanol–water, occurred maximally at tube 85, as shown in Fig. 3 (*b*).

The proportions of the various fractions were computed from the area under the curves (Collins & Shotlander, 1961) and the results are shown in Table 2.

DISCUSSION

Marinetti *et al.* (1958), in a study of the phospholipids of pig-heart-cell fractions, showed the presence of lysolecithin in the microsomes, although this material was not detected in the mitochondria and Macfarlane *et al.* (1960) failed to detect it in rat-liver-cell fractions. Marinetti *et al.* (1958) obtained lysolecithin in the proportion of 8% of the microsomal-lipid phosphorus, and in Fig. 3 the broken line represents distribution which would be expected if 8% of the lipid phosphorus had been lysolecithin. The experimentally determined curve has no peak in the precise position of lysolecithin

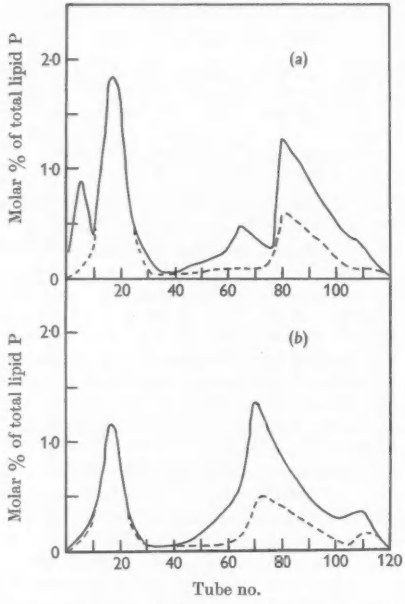

Fig. 1. Countercurrent distribution of the acetone-soluble-phospholipid derivatives in carbon tetrachloride–methanol–water. Every fifth tube was analysed for phosphorus (——) and dinitrophenyl groups (- - - - -). (*a*) Mitochondrial lipids (160 μg.atoms of phosphorus); (*b*) microsomal lipids (227 μg.atoms of phosphorus).

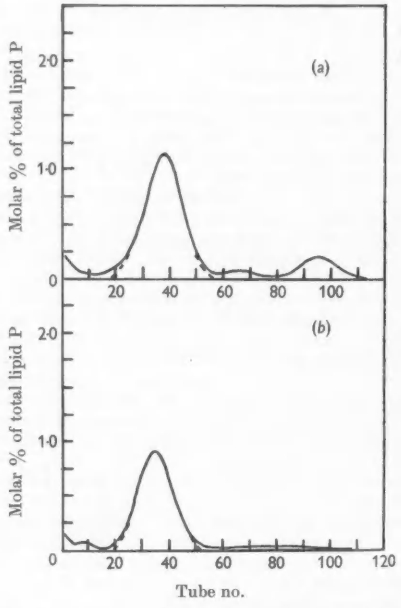

Fig. 2. Countercurrent distribution of the contents of tubes 1–35 of the distribution shown in Fig. 1. The solvent mixture was *n*-heptane–methanol and every fifth tube was analysed for phosphorus (——) and dinitrophenyl groups (- - - - -). (*a*) Mitochondrial lipids; (*b*) microsomal lipids. Results have been corrected for the material used in the analyses shown in Fig. 1.

and even if the small peak at tube 80 were lysolecithin it would represent no more than 0·5% of the total lipid phosphorus. The present work therefore does not support claims for the occurrence of lysolecithin in microsomal lipids although species differences cannot be ruled out.

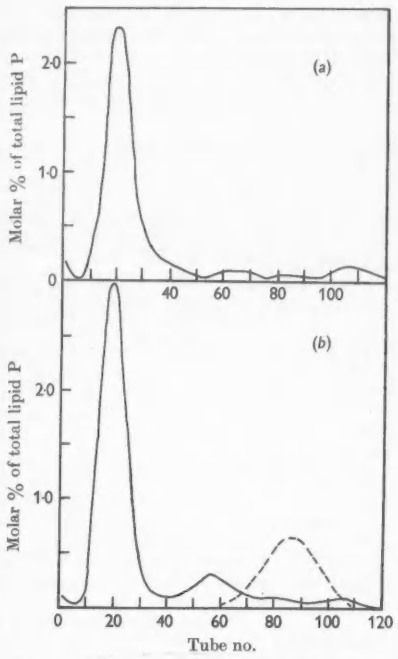

Fig. 3. Countercurrent distribution of the acetone-insoluble-phospholipid derivatives in carbon tetrachloride–chloroform–methylene chloride–methanol–water. Every fifth tube was analysed for phosphorus (——). (*a*) Mitochondrial lipids (92 μg.atoms of phosphorus); (*b*) microsomal lipids (212 μg.atoms of phosphorus) and the distribution of a sample of lysolecithin (-- -- -- --).

Collins (1959) found that three compounds occurred in small amounts in rat-liver lipids which resembled phosphatidic acid in that they contained neither choline nor amino bases. It was shown that one of these compounds (fraction Y) had a high radioactivity after incorporation of inorganic ^{32}P *in vitro*, but had a fatty acid to phosphorus molar ratio of 4. Hokin & Hokin (1958) and Hübscher & Clark (1960) found phosphatidic acid in animal tissues, although in amounts of less than 1%. Marinetti, Erbland, Albrecht & Stotz (1957) also presented evidence for the occurrence of a phosphatidic acid-like material but with an acyl ester/phosphorus molar ratio of 6. Thus the evidence is conflicting and does not exclude the possibility that both phosphatidic acids and bisphosphatidic acids may occur in animal tissues. Hence the material labelled 'phosphatidic acid' in Table 2 may be either of these materials or a mixture of both.

The fraction X described by Collins (1959) has been shown to be distributed in a similar manner to cardiolipin and this is strong evidence for the identity of this fraction. In microsomes the lipids contain a very small amount of cardiolipin-like material and of the 'phosphatidic acid', but in the mitochondria both fractions are well defined. The cardiolipin predominates and represents 5% of the total lipid phosphorus. The high content of phosphatidic acid-like material in mitochondria is in good agreement with the results of Marinetti *et al.* (1958) for pig-heart-cell fractions and of Macfarlane *et al.* (1960) for rat-liver-cell fractions.

Collins (1960) has presented evidence that the complex phospholipids are easily broken down by the analytical procedures in general use to produce diester phospholipids, which are identical with the classical phospholipids. It is also suggested that the finding of unusual compounds such as lysolecithin could be due to splitting of these complex phospho-

Table 2. *Phospholipids of mitochondria and microsomes*

(1) Present results; (2) Marinetti, Erbland & Stotz (1958) for pig-heart-cell fractions; (3) Macfarlane, Gray & Wheeldon (1960) for rat-liver-cell fractions. —, Signifies not detected.

	Phosphorus (molar % of total lipid phosphorus)					
	Mitochondria			Microsomes		
	(1)	(2)	(3)	(1)	(2)	(3)
'Phosphatidic acid'	2	13*	9†	1	2*	1†
'Cardiolipin'	5			1		
Phosphatidylethanolamine and phosphatidylserine	20	28	31	12	25	26
Complex phospholipids	37	—	—	39	—	—
Lecithin and sphingomyelin	31	42	51	39	58	62
Inositol and minor phospholipids	5	14	6	9	4	10
Lysolecithin	—	—	—	—	8	—

* Described as polyglycerolphosphatide. † Described as cardiolipin.

lipids. Collins (1960) has proposed that the complex phospholipids may be phosphate triesters and that this structure is consistent with the above-mentioned facts.

In the present study of the phospholipids of rat-liver mitochondria and microsomes it is apparent that the difference in the complex phospholipids in these two fractions is due to the presence of a component in microsomes which is absent from mitochondria. The fact that one component of the complex phospholipids seems to be associated with the endoplasmic reticulum raises speculation about the role of triester phospholipids in the functioning of the cell.

SUMMARY

1. The phospholipids from rat-liver mitochondria and microsomes have been examined by the countercurrent distribution of the dinitrophenylated and methylated derivatives.

2. The mitochondrial lipids contain a higher proportion of cardiolipin than do the microsomes.

3. The mitochondria lipids lack a component of the complex amino-phospholipids which is present in microsomes. No lysolecithin could be detected.

We wish to thank Miss H. Doery of the Commonwealth Serum Laboratory, Australia, for a gift of lysolecithin, Dr P. Plackett of the Animal Health Division, Commonwealth Scientific and Industrial Research Organization, for a gift of cardiolipin, the Standard Vacuum Refining Co. Aust. Pty. Ltd. for a gift of *n*-heptane and the National Health and Medical Research Council for financial support. One of us (V. L. S.) held a Commonwealth Post-graduate Award and a grant from the University of Melbourne. We wish to express our appreciation for the technical assistance of Miss T. B. G. Kaak.

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Mammalian Glycosidases

3. THE INTRACELLULAR LOCALIZATION OF β-GLUCURONIDASE IN DIFFERENT MAMMALIAN TISSUES*

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Kerr & Levvy (1951) and Walker & Levvy (1951) showed that a large part of the β-glucuronidase activity in water homogenates of mouse liver was associated with the cytoplasmic granules, and was apparently spread over particles of all sizes. The enzyme was fully active in water homogenates, and the fraction associated with the granules could be brought into a soluble state by incubation in acetate buffer, mechanical disintegration, and freezing and thawing, as well as by treatment with acetone or with the non-ionic, surface-active agent Triton X-100 (Rohm and Haas Co.). Walker (1952) extended this work to mouse liver homogenized in sucrose solution (0·25 M), and found that nearly all the enzyme was then associated with the cytoplasmic granules. The enzyme within the granules now displayed only a fraction of its total activity, owing apparently to slow diffusion of the substrate into the particles, since the activity rose when the substrate concentration was increased. The enzyme could be released by the measures already described, and, in addition, by dilution of the medium with water. These methods made the enzyme fully active, the most convenient being the use of Triton X-100. It was concluded that semi-permeable membranes must play a considerable part in regulating the activity of the enzyme *in vivo*.

* Part 2: Conchie & Hay (1959).

As a prelude to similar work on other mammalian glycosidases, a comparative study has now been made of the localization of β-glucuronidase in white-mouse liver, kidney, spleen and cancer tissue. Two tissues from C3H mice, together with pig liver, were included to observe whether the low β-glucuronidase activity in these organs (Morrow, Carroll & Greenspan, 1951; Conchie, Findlay & Levvy, 1959*a*) resulted in a different distribution of the enzyme within the cell.

EXPERIMENTAL

Materials

Phenolphthalein β-D-glucuronide was prepared biosynthetically as described by Levvy & Marsh (1959).

The adult white mice were from our Institute colony (Tyler's Original: Mill Hill Colony). The C3H strain was obtained from the Institute of Physiology, Glasgow University. Only male-mouse kidney was used because of the sex difference in the β-glucuronidase activity of this organ (Conchie, Findlay & Levvy, 1959*b*).

Mouse tumours, Ehrlich and T2146, were kindly supplied by Dr A. J. Carr, Department of Pathology, University of Aberdeen, and the S37 tumour by Professor P. C. Koller, Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3. All tumours were maintained in our own colony of white mice by subcutaneous transplantation. Suspensions for transplanting were made by grinding the tissue by hand in a glass homogenizer with a diameter clearance of 0·6 mm.

Pig liver was obtained immediately on slaughter from Robert Lawson and Sons (Dyce) Ltd. On removal, the liver was stored under 0·25M-sucrose solution at 0° and used as soon as possible.

Preparation of homogenates

Tissues were homogenized in a cooled all-glass homogenizer of the Potter–Elvehjem type with a diameter clearance of 0·23–0·43 mm. between the pestle and the tube, driven by an electric motor, average speed 1500 rev./min. For the preparation of sucrose homogenates, tissues were washed with ice-cold 0·25M-sucrose, pressed between pieces of hardened filter paper, weighed and homogenized in 0·25M-sucrose. The homogenates were used as soon as possible. Periods of homogenizing were varied, as stated in the text.

Water homogenates were prepared by homogenizing for ½ min. or 1 min. as stated below. The preparations were made 0·1M with respect to acetic acid–NaOH buffer, pH 5·2, and kept for 30 min. before use. This delay permitted full activity to be reached, and avoided the small variable activation of water homogenates by Triton X-100 sometimes observed by Walker & Levvy (1951, 1953).

Enzyme assay

Assays were carried out as described by Levvy (1952), with 0·05M-acetic acid–NaOH buffer, pH 5·2. In experiments involving the measurement of the activity of untreated homogenates in 0·25M-sucrose solution, the incubation buffer for all assays contained 0·25M-sucrose, final concentration. For measurement of the total activity of sucrose homogenates, Triton X-100 (0·1%, w/v) was also included (Walker, 1952). The final supernatant from centrifugal fractionation (see below) did not require Triton X-100 to display full activity. The addition of the non-ionic surface-active agent Triton X-100 (0·1%, w/v) to sucrose homogenates of all the tissues resulted in the display of activities equal to those obtained by homogenizing the corresponding tissues in water, after due allowance had been made for the small inhibitory effect of 0·25M-sucrose on β-glucuronidase activity.

Because of their low activity, pig-liver preparations were incubated with the substrate for 4 hr. (Tables 2 and 3) or 17 hr. (Table 1), instead of 1 hr. as with the other tissues, in order to get accurate readings. The activity did not appear to alter on prolonged incubation.

RESULTS

Variation in homogenizer clearance

The distribution of β-glucuronidase activity in the fractions obtained from various tissues (see below) depended upon the clearance between the pestle and tube of the homogenizer. Potter (1955) found that the optimum clearance for each tissue was different, and the critical fit of the homogenizer could be determined only by experience, depending upon the purpose of the experiments. The tissues studied here, with the possible exceptions of the mouse tumours and pig liver, were all of types which have been found by Potter to require a similar homogenizer clearance. As a result of preliminary experiments, homogenates were prepared in homogenizers with a diameter clearance of 0·23–0·43 mm. between the pestle and the tube. When the clearance became greater than 0·43 mm., the homogenizer was discarded in order to keep the time relationships constant. Within the above clearances, reproducible results were obtained. Homogenizers with a smaller clearance tended to destroy cytoplasmic particles as soon as the cell was disrupted, since there was an immediate appearance of an abnormally large activity in the soluble enzyme fraction.

Fractionation procedure

This was a modification of that described by Hogeboom (1955) for liver-cell components, all separations being done at 0°. The 0·25M-sucrose homogenate (10 ml.; 5% moist tissue) was layered over 0·34M-sucrose (10 ml.) and centrifuged at 700*g* for 10 min. in a MSE Minor centrifuge. After removal of the supernatant with a capillary pipette, the residue was suspended in 0·25M-sucrose (2–3 ml.) and again centrifuged at 700*g* for 10 min. The washed residue, consisting mainly of unbroken cells and nuclei, was called fraction I.

Supernatant and washings were centrifuged at 5000*g* for 10 min. in a MSE Superspeed 25 centrifuge, the entire supernatant, including the

'fluffy layer', was removed with a capillary pipette, and the residual pellet washed by resuspension in 0·25 M-sucrose (5–6 ml.) by using the homogenizer for a brief period at low speed. After recentrifuging, washing was repeated, the final washed residue, which contained the larger cytoplasmic particles, being called fraction II.

The combined washings and supernatant were made 0·1 M with respect to acetate, with acetic acid–sodium hydroxide buffer, pH 5·2, containing 0·25 M-sucrose, and centrifuged at 1500*g* for 10 min. The supernatant was removed, and the residue, after washing with 0·25 M-sucrose (10 ml.) containing 0·1 M-acetic acid–sodium hydroxide buffer, pH 5·2, again centrifuged, giving fraction III (Walker & Levvy, 1951; Walker, 1952), the small cytoplasmic granules.

The final supernatant and washings, which were particle-free when examined microscopically, were called fraction IV. A number of assays were also carried out on homogenates fractionated without the aid of acetic acid–sodium hydroxide buffer, fraction III being obtained by centrifuging the supernatant from fraction II at 60 000*g* for 60 min. Identical results were obtained with the two procedures and the acetate precipitation method was adopted as a general fractionation technique. By either procedure, fraction IV is defined as 'soluble' enzyme.

For assay, all fractions were suspended in appropriate volumes of 0·25 M-sucrose, and assayed in the presence of Triton X-100 (0·1 %), the resulting activity being expressed as a percentage of the activity obtained when the unfractionated homogenate in 0·25 M-sucrose was assayed in the presence of Triton X-100.

Table 1. *Distribution of enzyme activities in sucrose homogenates after fractionation*

For details of homogenization and fractionation procedures see text. Results are expressed as a percentage of the activity of a sucrose homogenate: all assays were done in the presence of Triton X-100 (0·1 %).

		Period of homogenization				
	Fraction	'Hand ground'	¼ min.	1 min.	3 min.	4 × ¼ min.
Mouse liver	I	49	11	8	7	8
	II	24	34	30	19	32
	III	19	37	31	27	36
	IV	6	9	18	32	14
	Recovery	98	91	87	85	90
Mouse kidney	I	40	32	21	19	19
	II	38	30	19	16	26
	III	13	17	14	13	26
	IV	9	16	39	43	24
	Recovery	100	95	93	91	95
Mouse spleen	I	65	21	14	15	19
	II	10	5	3	2	5
	III	12	12	9	5	12
	IV	18	62	75	79	60
	Recovery	105	100	101	101	106
S 37-mouse tumour	I	27	23	19	7	10
	II	17	16	15	11	16
	III	10	10	10	12	14
	IV	45	51	52	64	55
	Recovery	99	100	96	94	95
C 3 H-mouse liver	I	62	30	24	16	25
	II	11	21	16	12	20
	III	20	28	24	28	37
	IV	2	14	35	50	15
	Recovery	95	93	99	106	97
C 3 H-mouse kidney	I	30	14	9	3	1
	II	13	10	6	1	10
	III	44	41	28	9	51
	IV	13	28	50	81	29
	Recovery	100	93	93	94	91
Pig liver	I	36	29	16	2	2
	II	28	42	24	8	35
	III	21	9	21	23	36
	IV	13	17	37	72	27
	Recovery	98	97	98	105	100

Effect on fractionation of varying the period of homogenizing

To ascertain the conditions which gave maximum breakdown of whole cells with minimum disruption of cytoplasmic particles, each tissue studied was subjected to homogenization for various periods of time, the fractionation procedure described above being carried out in each case. The results for a series of such determinations are shown in Table 1.

'Hand ground' homogenates were obtained by placing the tissue and sucrose solution in the homogenizer tube and depressing and raising the plunger by hand till an even suspension was obtained. Usually 10 strokes of the plunger were sufficient. An additional series of fractionations ($4 \times \frac{1}{4}$ min.) was also done, in which, after a $\frac{1}{4}$ min. period of homogenization, fraction I was separated and subjected to homogenizing for three further $\frac{1}{4}$ min. periods, the supernatant (fractions II, III and IV) being removed at the end of each period and pooled for further fractionation. The object of this procedure was to obtain maximum disintegration of the cells while subjecting the granules to the minimum risk of damage. No differences were observed when 1 mM-disodium ethylenediaminetetra-acetate was added to the sucrose solutions used for homogenization.

The 'hand ground' results show that, compared with other tissues, a very large percentage of the total β-glucuronidase activity in the tumour was found in fraction IV, the cytoplasm. Similar results were obtained with two other types of mouse tumour, Ehrlich and T2146. Mouse spleen also displayed a pattern of distribution different from the other tissues. After being homogenized for $\frac{1}{4}$ min., fraction I retained about the same percentage enzyme activity as in the case of the other tissues, but by far the greater part of th activity released from fraction I appeared in the cytoplasm.

The tissues studied varied to some extent in their rate of breakdown in 0·25 M-sucrose. Whereas with mouse liver homogenizing for $\frac{1}{4}$ min. was sufficient to break down the cells almost completely, other tissues were not always so rapidly disrupted. Prolonged homogenizing of the entire tissue did bring about a decrease in the activity of fraction I, but there was some loss of enzyme activity from fractions II and III, indicating breakdown of the granules, and a progressive increase in fraction IV. When fraction I alone was subjected to repeated homogenization, further breakdown of this fraction was obtained with minimum loss of activity in fractions II and III, and this presumably gave a truer comparison of the distribution of enzyme activity in tissues which were variable in their resistance to homogenization.

Enzymic activities in sucrose homogenates

Walker (1952) showed that homogenates of mouse liver in 0·25 M-sucrose solution only displayed about 60% of their total β-glucuronidase activity on direct assay. Similar phenomena were now found with sucrose homogenates of other tissues, though the percentage of total activity displayed varied with the tissue.

Table 2 shows the activities of the various tissues in sucrose solution, expressed as a percentage of the activity measured in the presence of Triton X-100, and the percentage of the total tissue activity that was found in the soluble fraction in sucrose and water homogenates. Particularly high percentage activities in the whole sucrose homogenates were observed with white-mouse spleen, C3H-mouse liver and pig liver.

Table 2. *Enzyme activities of tissue homogenates in 0·25 M-sucrose, and of the soluble fractions from sucrose and water homogenates*

Whole tissues were homogenized for the single periods shown. The whole sucrose homogenate was assayed in the absence of Triton X-100, and the results were expressed as a percentage of the total activity determined in the presence of Triton X-100. The soluble fractions were prepared by removing all particulate material with 0·1 M-acetic acid–NaOH buffer, pH 5·2, and the results are expressed as a percentage of the total activity in the homogenate. Total activities are expressed as μg. of phenolphthalein liberated/g. wet wt. of tissue in 1 hr.

	Period of homogenizing (min.)	Sucrose homogenate		Water homogenate.	
		Whole homogenate	Soluble fraction	Soluble fraction	Total activity
White-mouse liver	$\frac{1}{4}$	51	12	52	2440
kidney	$\frac{1}{4}$	59	24	67	2090
spleen	$\frac{1}{4}$	86	50	92	4050
S37 tumour	1	57	47	75	2600
T2146 tumour	1	—	52	82	2870
Ehrlich tumour	1	52	47	84	2120
C3H-mouse liver	$\frac{1}{4}$	85	15	87	228
kidney	$\frac{1}{4}$	69	21	56	870
Pig liver	1	85	25	83	138

Whereas water homogenates of all the tissues were fully active without Triton X-100 only a proportion of this activity appeared in the soluble fraction, and this fraction varied from tissue to tissue. A high figure under this heading appeared to depend upon either (*a*) ready penetration of the granules, as betrayed by a high activity for the sucrose homogenate in absence of Triton X-100, or (*b*) a high figure for the cytoplasmic fraction obtained from the sucrose homogenate. The mouse tumours were an example of the latter, the soluble fraction containing a much greater percentage of the total enzyme activity in the water homogenate than that of white-mouse liver or kidney. This factor must have led to exaggeratedly high figures for cancer tissue in early assays of β-glucuronidase carried out with the soluble fraction from water homogenates (see review, Levvy & Marsh, 1959).

Sucrose homogenates were diluted with 6 vol. of water. The soluble fraction then reached the figure for the same tissue homogenized in water, whereas the total activity (assayed in absence of Triton X-100) was within 10% of full potential activity of the tissue (Triton X-100 present).

Effect of incubation in acetate buffer on the release of enzyme

Table 3 gives details of the results obtained when sucrose homogenates were incubated for various periods of time in the presence of 0·1 M-acetic acid–sodium hydroxide buffer, pH 5·2, the tonicity of the medium being kept constant. At the end of each incubation period both the activity of the whole homogenate and that of the soluble enzyme (obtained after centrifuging the homogenate at 1500*g* for 10 min.) were assayed and expressed as a percentage of the activity obtained on assaying the homogenate in presence of Triton X-100. No inactivation was observed even after incubation for 22 hr.

In all cases there was a considerable release of enzyme, tending to a maximum at the figure for soluble enzyme in a water homogenate (cf. Table 2). Moreover, though in some cases the enzyme became more accessible to substrate, as was shown by higher percentage total activity figures, in no case did the activity level reach that of the fully activated enzyme. In both respects, the time relationships varied from tissue to tissue. The results suggest that there was no great change in the location of the enzyme or in its accessibility to substrate under our assay conditions.

DISCUSSION

In all the tissues examined, particulate β-glucuronidase was distributed between the large and small granules. This is in accord with observations on rat liver (Gianetto & de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), where part of the enzyme was found in the microsomes, in which it displayed the same 'latency' as the 'lysosomal' fraction. In the microsomes there is no reason to believe that β-

Table 3. *Effect of incubation at* 37° *and* pH 5·2 *on the activity of a tissue homogenate in sucrose solution and of the particle-free supernatant prepared from it*

Homogenates prepared in 0·25 M-sucrose were incubated for various periods of time at 37° in 0·1 M-acetic acid–NaOH buffer. Assays were carried out in a buffer containing 0·25 M-sucrose. Results are expressed as a percentage of the activity of the corresponding whole homogenate assayed in the presence of Triton X-100. The whole tissues were homogenized for the single periods shown.

	Period of homogenizing (min.)		Period of incubation (hr.)					
			0	½	1	2	4	22
Mouse liver	½	Whole homogenate	45	46	47	53	74	89
		Soluble fraction	7	8	8	22	56	67
Mouse kidney	½	Whole homogenate	52	53	46	70	74	91
		Soluble fraction	15	20	35	41	46	66
Mouse spleen	½	Whole homogenate	87	88	87	86	87	88
		Soluble fraction	57	71	77	81	85	83
Mouse S 37 tumour	1	Whole homogenate	43	52	55	61	—	65
		Soluble fraction	30	28	34	40	—	68
Mouse Ehrlich tumour	1	Whole homogenate	52	49	48	53	—	59
		Soluble fraction	45	40	47	51	—	69
C3H-mouse liver	½	Whole homogenate	85	81	84	85	89	91
		Soluble fraction	31	45	61	65	74	83
C3H-mouse kidney	½	Whole homogenate	64	73	72	84	83	89
		Soluble fraction	21	48	49	53	55	63
Pig liver	1	Whole homogenate	91	89	88	82	88	86
		Soluble fraction	28	38	44	54	70	77

glucuronidase should be any less active *in vivo* than, for example, the microsomal enzyme glucuronyl transferase (Dutton & Storey, 1954; Strominger, Kalckar, Axelrod & Maxwell, 1954) which synthesizes β-glucuronides. In the same way, there appear to be no grounds as regards physiological activity for distinguishing between β-glucuronidase and mitochondrial dehydrogenases that are known to exhibit latency, such as succinic dehydrogenase (Singer & Lusty, 1960), and glutamic, malic and D-β-hydroxybutyric dehydrogenases (Bendall & de Duve, 1960). Even though in short-term experiments *in vitro* the activity of an enzyme is decreased when the integrity of the granules is preserved, there is no reason to believe that substrate equilibrium across the granular membrane could not be established eventually *in vivo*. Moreover, the possibility of a steady secretion *in vivo* of enzyme from the granules into the cytoplasm cannot be excluded. 'Latency' might thus be regarded as representing a device for regulating the catabolic activity of the healthy cell, that breaks down on the death of the cell (de Duve, 1959; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959; Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960).

Whereas mouse kidney resembled mouse liver in that the bulk of the enzyme was situated within granules, and thereby markedly decreased in activity in 0·25 M-sucrose solution, a large part of the enzyme in spleen and cancer tissue was free in the cytoplasm. It would therefore appear that the β-glucuronidase activity of the latter tissues must be markedly enhanced *in vivo* in comparison with tissues like kidney and liver. This finding opens up interesting possibilities with regard to the use of saccharo-1 → 4-lactone (Levvy, 1952) as an inhibitor of the enzyme in pathological conditions (Boyland & Williams, 1956). It has an obvious bearing on the suggestion that cancer drugs might be usefully administered as their β-glucuronides (Danielli, 1950) with a view to selective hydrolysis at the desired site of action. Carcinogen β-glucuronides in the circulation could also be subject to selective hydrolysis at different sites.

No matter how elaborate the centrifugal analysis of the distribution of an enzyme in homogenates may be, it must be recognized that the homogenizing technique may itself introduce artifacts. Liver is very nearly an ideal tissue for studying the intracellular localization of an enzyme by centrifugal methods, in that the cells are almost completely fragmented before breakdown of the granules occurs, provided the homogenizer clearance is not too small (see fraction IV, Table 1). Kidney, on the other hand, behaved in this respect as though the cells were tougher than those of liver, whereas the granules were more fragile. In the tumours, both the cells and the granules appeared to be tougher than those of liver.

The low β-glucuronidase activity of the C3H-mouse tissues and of pig liver compared with most other strains of mice and other mammalian species (for review, see Levvy & Marsh, 1959) was not compensated for (as might have been anticipated) by an increase in the fraction of the enzyme free in the cytoplasm, but C3H-mouse liver and pig liver did show a greater accessibility of the granular enzyme to substrate, as well as greater release of the enzyme from the granules on incubation of the homogenate in 0·25 M-sucrose solution or on homogenizing the tissue in water A few experiments with female C3H kidney, done since this work was completed, suggest that the lower overall enzyme activity (about 30% of the male) is compensated for by greater penetration of the granules by substrate in sucrose homogenates. The fact that homogenizing a tissue in water releases only a fraction of the β-glucuronidase activity from the granules has been noted (Kerr & Levvy, 1951; Walker & Levvy, 1951), and may be explained in two ways, either by assuming that part of the enzyme within the granule is bound more firmly than the rest, or that there are within the cell two types of particle, differing in their fragility.

SUMMARY

1. A study has been made of the intracellular localization by differential centrifuging of β-glucuronidase in 0·25 M-sucrose homogenates of adult white-mouse liver, kidney, spleen and cancer tissue, adult C3H-mouse liver and kidney, and pig liver.

2. In all tissues except mouse spleen and cancer tissue most of the enzyme was associated with the cytoplasmic granules, within which it displayed only a fraction of its total activity. In mouse spleen and cancer tissue a large part of the enzyme was free in the cytoplasm, and most of the remainder was associated with the cytoplasmic granules.

3. The remarkably low β-glucuronidase activity of pig liver and C3H-mouse liver and kidney as compared with white mice was not compensated for by any increase in the amount of the enzyme free in the cytoplasm, but the enzyme within the granules was more freely accessible to substrate.

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Electrical Pulses and the Potassium and Other Ions of Isolated Cerebral Tissues

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The differential distribution of sodium and potassium salts which is found in most animal tissues is of special significance in neural systems, for here the substances take part in the rapid ion movements associated with the nerve impulse. Loss of K^+ ions and gain in Na^+ ions on electrical stimulation of peripheral nerve is well documented (Keynes, 1951; Hodgkin, 1958), and it is a general impression that a major part of energy utilization by cerebral tissues is connected with the work performed in maintaining their characteristic ion distribution. When cerebral tissues are removed from an animal, promptly sliced and placed in media similar in content of Na^+ and K^+ ions to the extracellular fluids of the body they lose K^+ ions and gain Na^+ ions and water. Respiration in glucose is, however, associated with restoration of a large part of the K^+ ions and with extrusion of Na^+ ions (Terner, Eggleston & Krebs, 1950). Studies with isotopically-labelled potassium (^{42}K) salts showed that this was accompanied by a considerable turnover of the K^+ ions of the slices, of approximately 3·5–4 % of the tissue content/min. (Krebs, Eggleston & Terner, 1951).

The metabolism of isolated cerebral tissues in media similar to those employed in studying potassium movement, is affected by applied electrical pulses; energy-rich phosphates of the tissue are diminished and energy-yielding reactions accelerated (McIlwain, 1951, 1959). The content of Na^+ and K^+ ions and the exchange of K^+ ions have now been examined in electrically stimulated tissues. The tissues' respiratory and glycolytic responses to pulses have already been shown to be dependent on the addition of Na^+ ions to the medium in which they are incubated (Gore & McIlwain, 1952), and a similar dependence on K^+ ions is reported below.

EXPERIMENTAL

Ion movements

Tissues and electrodes. The procedures described by McIlwain (1960, 1961) were followed. Guinea pigs were used throughout the studies; they were stunned by a blow on the neck, killed by bleeding and the brain was removed in less than 3 min. The cerebral cortex was sliced to an average thickness of 0·35 mm. with a blade and recessed glass guide, weighed after draining on glass and placed in vessels which were incubated at 37·5° within 30 min. of the animal's death. Electrical pulses were supplied from the generator of Ayres & McIlwain (1953) to silver-grid electrodes at 100/sec., and were condenser pulses of peak potential 10v and time constant 0·4 msec.; for choice of these conditions see McIlwain (1954).

Ion movement and tissue composition. These studies were carried out in the rapid-transfer apparatus of Heald & McIlwain (1956) with electrodes with nylon gauze (McIlwain, 1960) and tissue slices of 80–100 mg. fresh wt. in 5 ml. of bicarbonate medium of composition (mM): NaCl, 120; KCl, 4·8; KH_2PO_4, 1·2; $MgSO_4$, 1·3; $CaCl_2$, 2·8; glucose, 10; $NaHCO_3$, 26. The initial content of Na^+ ions in the medium was thus 146 mM; its K^+ ion content was 6·0 mM and Cl^- ion content 130·4 mM. The medium was equilibrated with O_2+CO_2 (95:5, v/v), which was maintained in the vessels during incubation (Fig. 1).

In typical experiments, six weighed tissues were mounted in electrodes, incubated for 30–60 min., pulses were applied to certain of the tissues for periods of 10 sec.–30 min. and the electrode carrying its tissue was rapidly transferred to a second vessel for rinsing or extraction. Additional procedures adopted with ^{42}K are described below.

Rinsing and extraction. During the rapid transference of tissues for analysis, adhering fluid is inevitably also transferred; the effect of this on apparent tissue composition has previously been computed by calculation or minimized by a quick rinse in the cold fixing agent to be used in a subsequent extraction (Kurokawa, 1960). Because of the preponderance of Na^+ ions in the incubating fluids, rinsing is especially important in the present experiments, and has been appraised as shown in Fig. 2. Cold 0·3 M-sucrose was chosen as an innocuous sodium-free medium and slices were released from the holder into an adjacent dish containing 100 ml. of the sucrose; after defined times they were picked from the dish with a mounted bent platinum wire and placed in test tubes containing 1 ml. of conc. HNO_3 or trichloroacetic acid for analysis. The minimum time occupied by the transference was 3 sec., and in this time a sudden loss occurred in sodium and chloride, without appreciable change in potassium. After 3 sec. all three substances changed only slowly, and thus ample latitude was given for satisfactory transfer. Apart from the experiments of Fig. 2, all transferences were made in 3–4 sec.

Our interpretation of the change during 3 sec. in sucrose is that it represents loss of adhering medium (note that the apparent sodium and chloride concentrations at zero time, when referred to unit weight of the pre-weighed tissue, are greater than those of the medium) and that loss of the substances determined, even from the extracellular spaces of the tissue, is relatively slow.

Experiments with ^{42}K. $^{42}K_2CO_3$ was received from the Atomic Energy Research Establishment, Harwell; usually

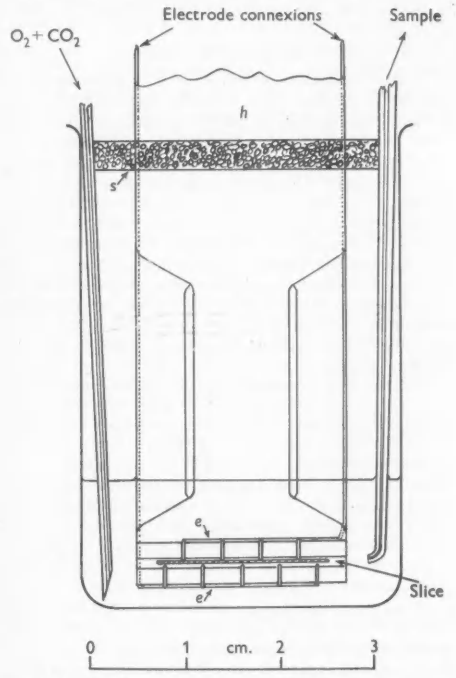

Fig. 1. Incubation and sampling in the quick-transfer apparatus. The slice is within the electrodes *e*, which form the lower parts of the holder *h*. The holder carries the sponge-rubber seal *s*, which enables the chosen gas atmosphere to be maintained in a 20 ml. beaker. For sampling, the capillary pipette is inserted at the position shown; for full details of the holder, its electrodes and thermostat, see Heald & McIlwain (1956) and McIlwain (1960).

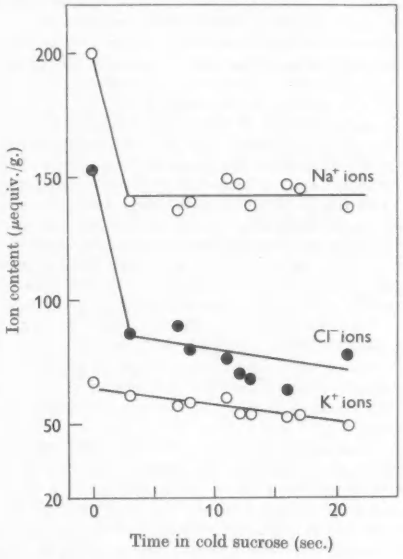

Fig. 2. Rinsing cerebral slices in cold sucrose. Tissue slices in quick-transfer electrodes were incubated in glucose–bicarbonate medium in quick-transfer electrodes for 30 min. and then were either released immediately into 3% (w/v) trichloroacetic acid (giving the values quoted at zero time) or released into 0·25 M-sucrose at 0° and, after the periods shown, transferred to the trichloroacetic acid. Each vertical group of three points represents one slice.

two experiments were performed with one sample, the first as soon as practicable after its removal from the pile (about 12 hr.) and the second on the following day. The sample (100 mg.) was dissolved in 5 ml. of water and brought to pH 7·4 with 0·1 N-HCl (approx. 0·3 ml.). All the KCl of a batch of the bicarbonate medium described above was replaced by the ^{42}KCl to make a stock ^{42}K–bicarbonate medium. In the first day's experiments, the initial incubation medium was prepared from 1 ml. of the stock ^{42}K–bicarbonate medium plus 4 ml. of ordinary bicarbonate medium; on the second day the two solutions were used in the proportion of 2:3.

In each case, experiments comprised an initial incubation of 30 min. at 37·5°, after which the slice, still in its holder, was quickly rinsed in 5 ml. of non-radioactive glucose–bicarbonate medium also at 37·5°. With its holder it was then placed in a further 5 ml. of ordinary glucose–bicarbonate medium in a beaker (*A*) of the quick-transfer apparatus and with an O_2+CO_2 atmosphere. Here incubation was continued and samples of 0·05 or 0·1 ml. of the medium were taken by capillary pipettes at the intervals indicated in the individual experiments. By inserting the capillary pipette through the sponge-rubber seal of the quick-transfer apparatus, as shown in Fig. 1, on the side opposite to that which receives the gas supply, it was possible to maintain the O_2+CO_2 atmosphere during sampling.

During this period of incubation and sampling, electrical pulses were applied to some vessels. At its termination, the slices were successively released to cold sucrose and after 3 sec. were ground in 10 ml. of 3% trichloroacetic acid. Dilutions of the tissue suspension, and of the incubation medium, were placed in 10 ml. tube counters (M-6H of 20th Century Electronics Ltd.) and radioactivity was determined with an Ekco scaler model N530F.

The radioactivities of each sample (medium and tissue) from a given beaker at stage *A* of the experiment were counted in succession during a period of up to 30 min. These counts were corrected for radioactive decay during the counting period; corrections ranged from 0 to 3%. From these calculated values for radioactivity at the beginning of the counting period, addition in appropriate proportions gave a value for total radioactivity in each beaker. In Figs. 6 and 7, radioactivity in the fluid samples (corrected values) have been expressed as percentages of the total radioactivity in the appropriate beaker. This method of expression gave data with which one slice could be compared with another even though the amount of ^{42}K associated with the slices differed, which was the case when the slices differed in weight or were derived from different experiments.

In the experiments of Fig. 5, the course of ^{42}K movement during a period of 60 sec. was measured by transferring a slice from beaker *A* to one after another of a series of beakers each holding 5 ml. of bicarbonate medium at 37° and with an O_2+CO_2 atmosphere. The time occupied by each transference was less than 0·5 sec. and five transferences of one slice were made in the course of 1 min.; the several slices of an experiment were handled in succession. Results were calculated and expressed as already described.

Influx of ^{42}K into the slices was also measured in the quick-transfer apparatus. After the preincubation of 30 min. at 37·5° in 5 ml. of glucose–bicarbonate medium, 0·1 ml. of ^{42}K–glucose–bicarbonate medium was added by capillary pipette. Electrical pulses were applied to some slices of an experiment and after 5 min. the slices were transferred through sucrose to trichloroacetic acid as described above. Determination of radioactivity in the media and in tissue extracts was carried out as described above.

Respiratory response to pulses

The tissues for these experiments were prepared in media lacking or diminished in potassium salts. The media were based on the glucose–glycylglycine medium of Kratzing (1953) and prepared by replacing its KCl and KH_2PO_4 by equimolar amounts of sodium salts; small additions of KCl were made in instances described in the individual experiments. Slices obtained as described above were floated from the cutting blade and guide into the potassium-deficient medium, trimmed to size (approx. 45 mg.), drained on a glass surface, weighed, returned to a second dish of potassium-deficient medium and mounted in grid electrodes *H* and, with the electrodes, placed in conical manometric vessels *E* (Ayres & McIlwain, 1953) containing 3·5 ml. of the medium. Respiratory rates were measured manometrically; for further details see Fig. 8.

Analytical methods

Sodium and potassium. Slices, after brief rinsing in cold sucrose, were placed in a test tube containing 2·5 ml. of conc. HNO_3 or were ground in 2·5 ml. of 10% (w/v) trichloroacetic acid. After 12–24 hr. at room temperature, samples, usually of 0·25 ml., were diluted to 10 ml. and the sodium and potassium determined with a flame photometer (Evans Electroselenium Ltd., model A) with the appropriate filters. Standard curves were constructed from readings taken in the presence of the concentration of HNO_3 or trichloroacetic acid in the diluted samples (Collins & Polkinhorne, 1952), and with Na^+ and K^+ ions present in a molar ratio of 2:1. This is the approximate ratio of Na^+ to K^+ ions in typical samples, and the procedure is similar to that of Pappius & Elliott (1956*a*, *b*).

Chloride. This was determined by the method of Lowry *et al.* (1954) and Ames & Nesbett (1958) carried out on a larger scale. Excess of mM-$AgNO_3$ was mixed with 0·1 ml. of a 0·1N-HNO_3 extract or of the trichloroacetic acid extract of the tissue (see above) in a 2 ml. centrifuge tube. After 30 min. at room temperature the tube was centrifuged and, to 0·1 ml. of the supernatant, 3 ml. of the rhodanine reagent was added and the determination completed according to Lowry *et al.* (1954).

Inorganic phosphate and phosphocreatine. Slices from the quick-transfer apparatus, with or without the rinse in sucrose, were placed in 2·5 ml. of ice-cold trichloroacetic acid in a test tube homogenizer with a loosely fitting pestle, ground and the phosphates determined as described by R. J. Woodman & H. McIlwain (in preparation); methods were based on those of LePage (1949) and Heald (1954).

Tissue content of lactic acid. Slices were quickly rinsed in sucrose and extracted with trichloroacetic acid as for sodium and potassium determination, and samples of extract were transferred to $CuSO_4$, lime was added and the determination completed according to Barker & Summerson (1941).

RESULTS

Tissue composition and its change with pulses

Phosphocreatine, inorganic phosphate and lactic acid. In the Experimental section are described the incubation and transfer procedures adopted during the present experiments. In order to appraise the metabolic status of the tissue after these procedures, its phosphocreatine and inorganic phosphate contents were determined on several occasions during the course of the studies and a few measurements were made of tissue lactate. These constituents are sensitive to inadequacies in the supply of O_2 or glucose to the tissue, or to other abnormalities. The values obtained (Table 1) indicate satisfactory metabolic conditions (see McIlwain, Buchel & Cheshire, 1951; McIlwain, 1959). Moreover, the application of electrical pulses caused the loss of phosphocreatine and increase in inorganic phosphate typical of the normal tissue (Anguiano & McIlwain, 1951; McIlwain & Gore, 1951). Phosphocreatine has also been determined in tissues incubated in media containing ^{42}KCl prepared in the laboratory from $^{42}K_2CO_3$, and also found to be normal and to undergo a normal fall with pulses. The tissue lactate and its change with pulses are in accordance with the observations of McIlwain & Tresize (1956).

Sodium, potassium and chloride. (*a*) Normal content. The present method of handling cerebral tissue has been found to give a potassium content of 60–65 μmoles/g. wet wt., or 5–25 μmoles/g. greater than those quoted by Terner *et al.* (1950) and Pappius & Elliott (1956*a*, *b*) after incubation in comparable media. This may be due to small differences in the preparation of the tissue, to the mechanical support given to it during incubation or to the subsequent transfer procedures. The majority of the experiments of Terner *et al.* (1950) and of Krebs *et al.* (1951) were carried out with the further addition of glutamate, which affords still higher values for the potassium associated with a given weight of tissue. It has, however, been observed that this is accompanied by intracellular uptake of fluid (Pappius & Elliott, 1956*a*, *b*) and that the cellular concentration of potassium salts falls. Glutamate also lowers the phosphocreatine of cerebral tissues (McIlwain, 1952), renders their respiration and glycolysis largely unresponsive to electrical pulses and diminishes the resting potentials observable in polarized elements of the slices (Hillman & McIlwain, 1961). For these reasons glutamate has generally not been added in the present experiments, but its effects were studied in a few instances (see below).

The tissue absorbed much aqueous fluid on preparation and incubation, as has also been found by

Table 1. *Tissue composition and response to pulses*

Slices of guinea-pig cerebral cortex or liver weighing about 80 mg. were incubated in 5 ml. of glucose–bicarbonate medium in the electrodes designed for rapid transfer. After 30 min., condenser pulses were applied to some of the tissues. The slices were then transferred to 0·25 M-sucrose and plunged into cold trichloroacetic acid. They were extracted by grinding and the phosphates and ions were determined. (See the Experimental section for further details.) Values give the mean result ± S.D. of the number of experiments indicated in parentheses. Pulses of time constant 0·4 msec., peak potential 10 v, were applied at 100/sec. for 10 min. except in the instance marked with an asterisk, when application was for 1 min.

Tissue	Substance determined	Tissue content (μmoles/g. fresh wt.) No pulses	With pulses
Cerebral cortex	Phosphocreatine	1·5 ± 0·14 (8)	0·45 ± 0·15 (6)
	Inorganic phosphate	3·1 ± 0·3 (8)	4·0 ± 0·6 (6)
	Lactic acid	2·1 ± 0·5 (4)	8·1 ± 0·7 (4)
			4·0 ± 0·9 (4)*
	K	63 ± 5 (8)	47 ± 2·7 (6)
	Na	146 ± 11 (8)	152 ± 13 (6)
Liver	K	36·2 ± 6·4 (4)	35·5 ± 5·0 (4)

Table 2. *Tissue water, potassium, sodium and chloride*

Guinea-pig cerebral cortex was taken for analysis: (*a*) immediately after excision; (*b*) after cutting in glucose–bicarbonate medium; (*c*) and (*d*) after incubation as described in Table 1. Dry wt. was obtained after 2 hr. at 105° and reweighing at 3 hr.

Determination	Condition of tissue (*a*) Fresh	(*b*) Cut	(*c*) Incubated	(*d*) Incubated and pulses applied
Dry wt. (%)	20·3 ± 0·8 (4)	13·8 ± 1·1 (4)	11·9 ± 1·8 (5)	10·4 ± 1·7 (5)
K (μequiv./g. dry wt.)	550 ± 11 (6)	384 ± 64 (6)	442 ± 43 (8)	348 ± 29 (8)
Na (μequiv./g. dry wt.)	256 ± 8 (6)	862 ± 28 (6)	1015 ± 40 (5)	1036 ± 93 (6)
Cl (μequiv./g. dry wt.)	143 ± 14 (4)	406 ± 57 (4)	616 ± 48 (5)	478 ± 72 (6)

Leaf (1956) and Pappius & Elliott (1956*a*, *b*). In Table 2 the tissue dry weight and ionic constituents are shown, derived from experiments which include those of Table 1. Expressing the ionic constituents in terms of tissue dry weight, in comparison with the values obtained by Leaf (1956) using rat cerebral cortex, again shows tissue potassium to be well maintained on incubation. The sodium and chloride in the incubated tissue (*c*, Table 2) are also high, indicating the fluid absorbed to be comparable in composition to the medium rather than water. Thus comparison of the dry weights (*a*) and (*c*), Table 2, shows the uptake of 3·48 g. of water/g. dry wt. of tissue; the increase in chloride of 473 μequiv./g. dry wt. would be contained in 3·62 ml. of medium. Taking into account observations of Leaf (1956), Pappius & Elliott (1956*a*, *b*), Thomas & McIlwain (1957) and Tower (1960), this fluid may be concluded to be extracellular. The 3·62 ml. of medium would carry 530 μequiv. of sodium/g. dry wt. of tissue; the tissue acquires more sodium than this, as has been noted also by Leaf (1956) in cerebral tissues of the rat. Interpretation of this change, and of that in chloride, would require allocation of sodium and chloride to the intracellular and extracellular compartments of the tissue, which is not examined in the present study.

(*b*) Effect of pulses. Electrical pulses diminished the potassium content of cerebral tissues but not of similarly maintained slices of liver (Table 1). The change in guinea-pig cerebral cortex in 10 min. is seen to involve loss of 25 % of the tissue potassium, or a molar quantity very much greater than that in the other cerebral constituents which have been examined. Thus the change in inorganic phosphate or phosphocreatine was about 6 % of that in potassium. Expressed in terms of tissue dry wt., pulses are seen from Table 2 to cause the loss of an amount of potassium salts greater than the total quantity reassimilated on incubation *in vitro*. Certain constituents or metabolic activities in cerebral tissues have been found to differ in concentration or magnitude in slices cut from different depths in the cerebral cortex, and Table 3 shows an examination of potassium salts from this point of view. The samples represent two-thirds of the thickness of the cortex, and are seen to be uniform in potassium content; pulses in all cases caused loss of K^+ ions. Loss of K^+ ions on applying pulses is seen to be accompanied by a rather greater loss in Cl^- ions (Table 2); Na^+ ions changed little if at all (Tables 1 and 2). Appraising the changes in all three ions suggests that some sodium had entered the cells on application of pulses; specific examination of this is deferred.

The effect of pulses on tissue potassium proved to be largely independent of added oxidizable substrates (Table 4). In the absence of such substrates, the potassium content of the cortical slices was lower than with glucose; glutamate afforded concentrations similar to those in the presence of glucose, and the greatest potassium content was found with both glucose and glutamate, in agreement with Terner *et al.* (1950). In each of these four cases, pulses diminished tissue potassium by amounts of 14–23 μmoles/g.

Table 3. *Potassium content of slices from different depths in the cerebral cortex*

Slices designated 1 were 0·35 mm. thick and were cut from the outer convexity of the guinea-pig cerebral cortex; slices 2 were from the remaining block, and slices 3 were cut after slices 2. Incubation and other details were as described in Table 1, except that pulses were applied for 5 min. only.

Slice	Content of K after incubation (μequiv./g. fresh wt.) No pulses	With pulses
1	63±4·8 (8)	56·4±2·5 (5)
2	60±4·8 (8)	51·5±3·2 (4)
3	61±5·3 (8)	48·6±2 (5)

Course of change in tissue potassium

Net movement with pulses. Control observations during the preceding experiments showed the potassium content of cerebral slices to be constant between 30 and 60 min. of incubation in oxygenated glucose media. When observations were made at briefer intervals the stability was confirmed, but application of pulses brought about a discernible loss of potassium salts within 1 min. (Fig. 3). The

Table 4. *Effect of substrates and electrical pulses on tissue potassium*

Experiments were carried out and results are expressed as described in Table 1, except for changes in substrate.

Substrate (mM)	Tissue potassium (μequiv./g. fresh wt.) No pulses	With pulses	Change
None	48±2 (5)	25±2 (4)	−23
Glucose (10)	61±8 (35)	47±4 (8)	−14
L-Glutamate (20)	62±6 (3)	40±3 (3)	−22
L-Glutamate (20) and glucose (10)	87±8 (6)	71±10 (6)	−16

loss continued at a diminished rate until 10 min., after which little change occurred. These observations involved the analysis of many tissues, and the inherent variation in their potassium content precluded accurate estimation of the rate of potassium loss during the initial phase, though the loss in 1 min. was statistically significant, with $P < 0{\cdot}01$. The variation also made it unprofitable to attempt to examine shorter time intervals, or to analyse mathematically the course of change in potassium. However, the data of Fig. 3 imply an initial rate of loss of at least 300 μequiv. of potassium/g. fresh wt. of tissue/hr. during the first minute, and a continued loss of about 40 μequiv./g. fresh wt./hr. until, after 10 min., stability was approached.

Switching off pulses at this point was followed by a remarkably rapid reassimilation of potassium salts (Fig. 4). About 10 μmoles/g. of tissue were regained in 1 min., after which only a relatively small change occurred. Slices cut first from a hemisphere (outer slices) differed from those cut second or third in the concentration of potassium attained, and are indicated separately in Fig. 4 (see also Fig. 8). This gives an indication of the heterogeneity of cerebral tissues which is important in appraising the findings (see Discussion).

Efflux of ^{42}K. Potassium movements during brief periods have been examined by using the isotope ^{42}K. In these experiments preincubation of the tissue took place in media containing ^{42}KCl, and after 30 min. the tissue was rinsed and incubated in a medium of the same composition except that it contained unlabelled potassium salts. Without application of pulses, ^{42}K rapidly appeared in the medium during incubation, indicating that tissue potassium was undergoing considerable exchange with that of the medium even while its total quantity was stable. The initial rate of efflux corresponded to 11 % of the tissue ^{42}K/min., or 400 μequiv./g. of tissue/hr. (Fig. 5).

When condenser pulses were applied to tissue preincubated with ^{42}K, efflux of the isotope increased by 60–80 % of its control value (Fig. 5). This increased rate corresponded to 600–750 μequiv. of potassium/g. of tissue/hr. and was established within the first 10 sec. application of pulses, despite its involving diffusion of ^{42}K from the tissue to the medium. In the experiment of

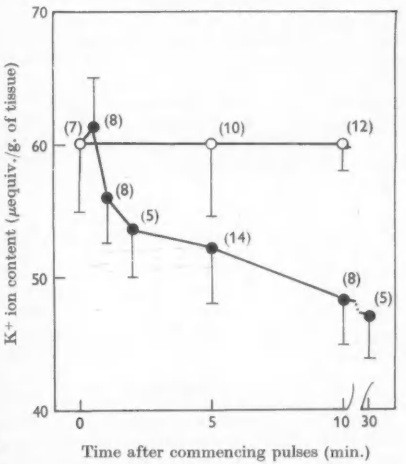

Fig. 3. Loss of potassium from guinea-pig cerebral cortex subjected to electrical pulses. The slices were in the quick-transfer apparatus and, after a preliminary incubation of 30 min., alternating condenser pulses of time constant 0·4 msec. and peak potential 10v were applied at 100/sec. Slices were then floated into 0·25 M-sucrose and rapidly transferred into trichloroacetic acid. Potassium was determined by flame photometry; points give the contents from the number of slices indicated in parentheses; vertical lines indicate the S.D. of the means. Differences between the potassium content of slices at zero time and after 1 and 2 min. of pulses showed $P < 0{\cdot}01$; the tissue exposed to pulses for 5 and 10 min. also differed from their controls without pulses, with $P < 0{\cdot}01$. Outer slices: ○; inner slices, ● (see text).

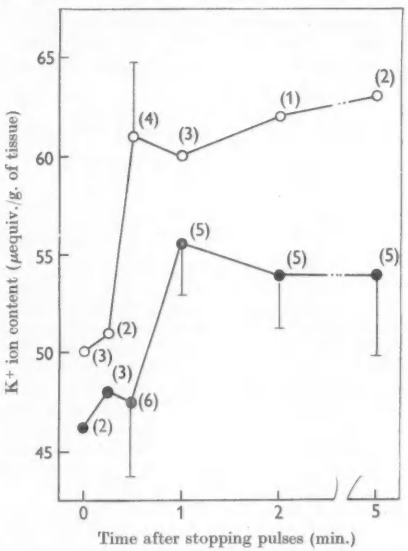

Fig. 4. Reassimilation of K^+ ions after stimulation for 10 min. Condenser pulses were applied for 10 min. and, at appropriate intervals after stopping the pulses, slices were floated into 0·25 M-sucrose; other details were as given in Fig. 3.

Fig. 5 (*a*), pulses were applied for 10 sec. only. This brief application of pulses doubled the rate of efflux both during and subsequently to their application, indicating an increased efflux of the isotope even during the period of recovery of total tissue potassium.

The efflux of ^{42}K induced by pulses was found to be relatively independent of the metabolic status of the tissue. In the absence of glucose (Fig. 6) the unstimulated efflux was greater than in the normal medium, and was further increased by applied pulses. Expressing the efflux with pulses as a percentage of that without pulses gives, however, a greater proportional change in the presence of glucose; tissue content of total potassium was greater with glucose (Table 4), as in the experiments of Terner *et al.* (1950). Glutamate added to glucose-containing media also caused increased efflux of ^{42}K, and this again was further augmented by application of pulses (Fig. 7).

Influx of ^{42}K. Labelled potassium salts added to media in which cerebral tissues are incubated were shown by Krebs *et al.* (1951) to enter the tissue rapidly; with similar experimental arrangements and calculations with the present glucose–bicarbonate medium, the values of Table 5 were obtained. The rate of entry in normal tissues corresponded to 5·4 % of the tissue potassium/min. over the interval

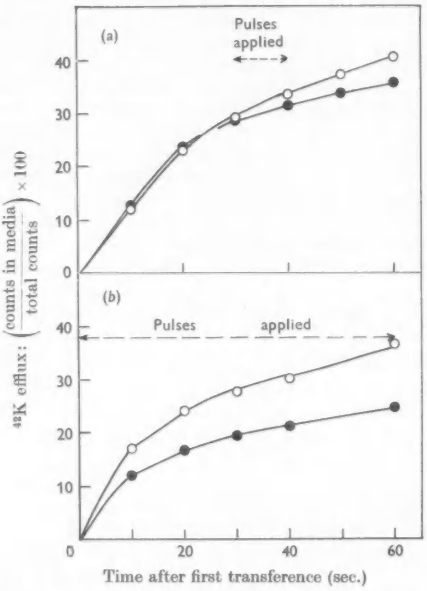

Fig. 5. Radioactive potassium efflux during brief periods. The slices, held in rapid-transfer electrodes, were incubated for 30 min. in 5 ml. of ^{42}K–glucose–bicarbonate medium (see the Experimental section). Tissue was then rinsed quickly and transferred at 10-sec. intervals from one to another of a series of beakers, each containing 5 ml. of fresh medium. The tissue was then floated into 0·25 M-sucrose and rapidly transferred into 10 ml. of trichloroacetic acid. Alternating condenser pulses (of time constant 0·4 msec., peak potential 10v at 100/sec.) were applied: (*a*) to slices (○) between 30 and 40 sec.; (*b*) to slices (○) between 0 and 60 sec. The filled-in points (●) give their respective controls without pulses. The radioactivity of each beaker and slice was counted and the efflux calculated as the sum of the counts in the media at different stages, as a percentage of the total counts from the slice and all media through which it had passed. Corrections for radioactive decay were made.

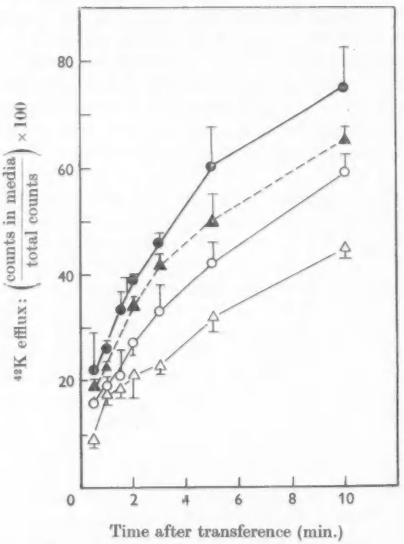

Fig. 6. Movements of ^{42}K from cerebral slices with and without glucose. The slices were preincubated at 37° in ^{42}K medium, rinsed and transferred quickly into 5 ml. of medium: ○, containing no glucose; ●, containing no glucose and with application of pulses; △, with glucose; ▲, with glucose and applied pulses. Samples (0·1 ml. of medium) were taken at appropriate time intervals. After 10 min. the tissue was floated into cold 0·25 M-sucrose and ground in trichloroacetic acid. Condenser pulses of the characteristics described in Fig. 5 were applied to the two sets of tissues indicated, for the whole 10 min. shown. Radioactivity of the samples was counted in a 10 ml. liquid tube counter, and expressed as total counts in the medium at that time. Corrections for radioactive decay and change in volume were made. Points give the amount of ^{42}K appearing in the media as the percentage of total ^{42}K added; vertical lines indicate S.D. of mean; each point corresponds to four determinations on different slices.

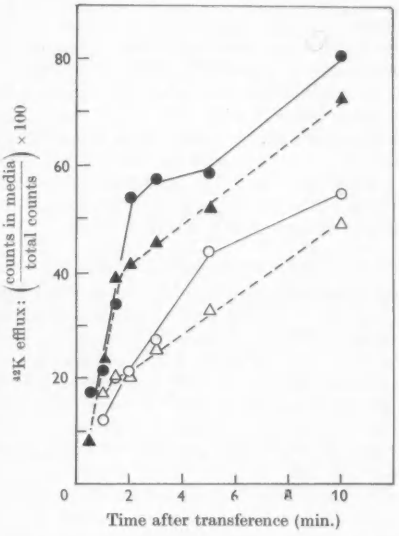

Fig. 7. Glutamate and the efflux of radioactive potassium from cerebral slices. Experimental conditions were as given in Fig. 6, except that glutamate (to 10 mM) was added to some vessels; △, glutamate alone; ▲, glutamate with pulses; ○, with glutamate and glucose; ●, with glucose, glutamate and pulses. Points give the average of two determinations of the percentage of the total ^{42}K appearing in the medium.

of 5 min. When pulses were applied, influx of ^{42}K calculated with this formula on the basis of the final K^+ ion content of the tissue rose to an average value of 12·6%. This took place despite a net movement of potassium in the opposite sense (Fig. 3). The net movement of potassium averages 2·5% of the potassium content/min. during the first 5 min. of pulses (Fig. 3). Although this makes the formula employed in calculation not strictly correct, increase in ^{42}K influx with pulses is a direct experimental observation (Table 5).

Dependence of metabolic response to pulses on potassium salts

Preliminary experiments showed that to lower effectively the concentration of potassium salts in slices of guinea-pig cerebral cortex it was necessary to handle the tissues throughout in media without added potassium salts. Even so, the media in which the tissues were first placed could accumulate appreciable quantities of K^+ ions. Thus in a typical experiment slices (0·7 g.) were cut and floated, from the blade and guide used in cutting, into a dish of 15 ml. of medium; here they were cut to size before weighing and placing in manometric vessels. In experiments in which the medium of the dish was initially potassium-free, it was found after receiving the slices to have become 2·6–2·8 mM in potassium salts; thus the concentration in this medium had risen to about half that of an ordinary Krebs–Ringer fluid (6 mM-K^+ ions). This implies a loss of 55–60 μmoles/g. of tissue from an

Table 5. *Electrical stimulation of the entry of* ^{42}K *into slices of guinea-pig cerebral cortex*

All slices were held in the quick-transfer apparatus and, after 30–40 min. preincubation, 0·1 ml. of stock ^{42}K medium was added. Condenser pulses of the characteristics given in Table 1 were applied to one of duplicate pairs of vessels, immediately on addition of the ^{42}K. After 5 min. further incubation, slices were released to sucrose and extracted with trichloroacetic acid as described in Table 1; radioactivity in tissues and incubation fluids was counted, and total potassium determined by flame photometer.

Wt. of slice (mg.)	Total potassium (μequiv.)		Radioactivity (counts/min.)			Calculated* rate of entry into slice	
	Slice	Medium	Initially (medium)	Finally (medium)	Finally (tissue)	(μequiv. of K/min.)	(% of K/min.)
			Slices without applied pulses				
88	5·1	40·6	11 710	11 292	418	0·349	6·8
79	5·3	40·8	10 734	10 408	326	0·285	5·4
129	7·1	40·6	8 628	8 391	237	0·247	3·5
91	5·2	46·2	37 707	36 760	947	0·280	5·4
66	3·6	47·0	36 371	35 466	907	0·206	5·7
Mean ± S.D.	—	—	—	—	—	—	5·4 ± 0·8
			Slices with 5 min. application of pulses				
77	4·6	47·8	38 116	36 752	1 364	0·416	9·1
68	3·6	46·5	37 534	36 369	1 165	0·378	10·5
76	3·4	46·0	36 765	35 229	1 536	0·590	17·4
79	3·6	46·4	37 405	36 034	1 371	0·475	13·2
Mean ± S.D.	—	—	—	—	—	—	12·6 ± 3

* Calculated according to Krebs, Eggleston & Terner (1951).

initial value of 100–110 μmoles/g., which is a loss comparable with that observed by Terner *et al.* (1950) to occur from cerebral tissues to media of normal potassium content.

Potassium content of incubated tissue

Tissue that had been prepared in the fashion described, and which had already lost potassium salts to potassium-free media, was found to lose further K^+ ions on incubation in a fresh batch of potassium-free medium. After incubation for 1–2 hr. slices (about 45 mg.) placed in 3·5 ml. of medium contained only 4–12 μmoles of potassium/g.

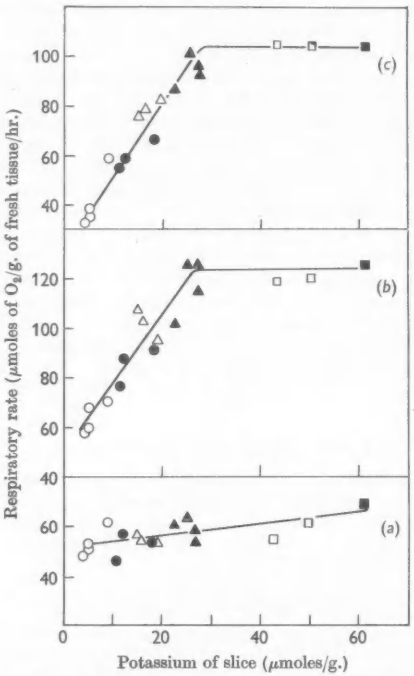

Fig. 8. Dependence of respiratory response to pulses, in slices of guinea-pig cerebral cortex, on potassium salts of medium and tissue. Slices were prepared and incubated in media which contained no added K^+ ions (○ and ●), or 1·1 mM-K^+ ions (△ and ▲) or 11 mM-K^+ ions (□ and ■). Slices ●, ▲ and ■ were from the outer convexity of the cortex and slices ○, △ and □ were inner slices. Respiration was measured in three successive periods: (*a*) up to 40 min. of incubation, without applied pulses; (*b*) from 40 to 70 or 80 min., when pulses of peak potential 10v, 0·4 msec. time constant, were applied at 100/sec.; (*c*) from 70 or 80 to 100 or 120 min., with continued application of pulses of the same characteristics. At the end of the experiments the slices were drained free of medium and their K^+ ion content was determined.

This is shown in Fig. 8 (reading the points only in relation to the abscissa), which indicates also that it is the outer slices which retain the greater quantities of potassium salts (see also Fig. 4). Tissues prepared in media without added potassium salts retained more K^+ ions if incubated as just described, but in media with added K^+ ions. Thus after 2 hr. in media 1·1 mM in K^+ ions their K^+ ion content was 15–30 μmoles/g. and in 11 mM-K^+ ions it was 40–50 μmoles/g., the outer slices in each case contributing the higher values.

Response to electrical pulses

Previous investigation had shown that tissue prepared in media of normal potassium content, incubated in media initially lacking potassium salts, was normal in its initial respiratory rate. Respiration increased in rate on applying electrical pulses to an extent similar to that observed in media of normal potassium content (Gore & McIlwain, 1952). Tissue prepared in media lacking potassium has, however, been found more susceptible. Before application of pulses, respiration tends to be low (Fig. 8*a*); when tissues were prepared in media without K^+ ions, values averaged 54 μmoles/g./hr. in place of 61 for tissues prepared in 1·1–11 mM-K^+ ions; this difference is just significant at $P < 0.05$.

The difference on application of electrical pulses is, however, much more striking (Fig. 8*b* and *c*). Tissues lowest in potassium salts (5 μequiv./g.) increased in respiration by only 10 μmoles of O_2/g./hr., whereas those with more than 25 μequiv. of K^+ ions/g. increased in respiratory rate by 60 μmoles of O_2/g./hr. Between the values of 5 and 25 μequiv. of K^+ ions/g. of tissue, the rate with pulses doubles, showing an approximately linear increase with increase in K^+ ion content. If the lines of Fig. 8 (*a*) and (*b*) are extrapolated to zero potassium content, they each cut the ordinate at 50 μmoles of O_2/g./hr., implying that no respiratory response would be expected in a tissue without potassium.

It is evident from Fig. 8 that tissues treated similarly with respect to the K^+ ions of the media in which they are handled can nevertheless differ in potassium content, either because of the depth in the cortex from which the slice was cut or for undetermined reasons. The relationship between potassium content and response to pulses is not disturbed by these variations; it holds for most of the subgroups shown by different points in the diagram: for example, among inner slices handled in media lacking K^+ ions (○) and among outer slices in media with 1·1 mM-K^+ ions (▲).

After pulses have been applied for 30–40 min., the respiratory rate of most tissues falls by 15–20 μmoles of O_2/g./hr. (Fig. 8*c*). Although in

Table 6. *Rates of change in tissue potassium*

Measurements followed 30–40 min. preincubation of guinea-pig cerebral cortex in glucose media; for other details see the sources quoted.

Movement	Source of data	Period measured	Rate (μequiv./g./hr.) No pulses	Rate (μequiv./g./hr.) With pulses
Net loss from tissue	Fig. 3	0–60 sec.	0	240
Net gain after pulses	Fig. 4	0–60 sec.	550–600	—
Efflux measured by ^{42}K	Fig. 5	20–60 sec.	400	600–750
Influx measured by ^{42}K	Table 5	0–5 min.	330	645

tissues under optimum conditions this implies a fall of only 10–15 % of their stimulated rates, in tissues from media without K^+ ions the fall can mean that pulses cause a decrease, rather than an increase, in respiratory rate. This is seen to occur when the tissue K^+ ion content is about 5 μmoles/g.

The lactic acid which accumulated in the media during the experiments of Fig. 8 was found to average $70 \pm 12(19)$ μmoles/g. of tissue, and not to show a marked dependence on the potassium content of the medium or of the tissue.

DISCUSSION

These experiments have given an opportunity of observing the course of the several distinct aspects of potassium movement which are listed in Table 6. These show major changes to be caused by the application of electrical pulses. The numerical values quoted in Table 6 refer in most cases to an arbitrarily chosen period in the first minute after applying or ceasing to apply pulses, or after adding the potassium isotope whose change is measured. Fuller information of the course of change is given in Figs. 3–5, most of which include periods in which the rates concerned are faster or slower than during the periods selected. Certain of the data give approximately linear relationships between the potassium content of tissues, at different times after initiating ion movement, and the square root of the time interval, a relationship described by Harris (1960), as shown by processes in which diffusion is a limiting factor. Diffusion is clearly involved in the passage of ions from the cells of the slice to intercellular fluids, and from these fluids to the external solution. Logarithms of potassium fluxes did not show a linear relationship to time. Closer analysis of the course of potassium movement has not been made, in view of the variety of potassium-containing structures, at both cellular and subcellular levels, which are contained in the tissue. This heterogeneity is discussed in more detail by Hillman & McIlwain (1961) in relation to the polarized elements observed (by micropipette electrodes) to be present in cerebral slices comparable with those used in the present study.

Ion movements and tissue metabolism

The movements of potassium now observed in cerebral tissues in response to pulses appear in several respects to be analogous to those occurring in peripheral nerve during excitation and recovery (see Hodgkin, 1958). Here the following points are relevant.

(i) No corresponding changes in ion movement were induced in liver slices by electrical pulses, although liver slices maintain intracellular concentrations of potassium salts several times those of the medium and display resting membrane potentials when examined with micropipette electrodes (Li & McIlwain, 1957). Cerebral but not liver tissues have previously been shown to respond to electrical pulses by increases in respiration and glucose utilization (McIlwain, 1951; Kratzing, 1951).

(ii) Nevertheless, the potassium efflux induced in cerebral tissues by stimulation is not secondary to these changes in respiration or in glucose utilization, for the potassium efflux occurs in media without glucose, and applied pulses do not increase respiration under these conditions (McIlwain, 1953). Loss of phosphocreatine from cerebral tissues also normally accompanies their electrical stimulation, but is not necessary to the efflux of potassium in response to pulses, for this efflux takes place under conditions in which the tissue has little or no phosphocreatine: in the absence of glucose or in the presence of both glucose and glutamate. These conditions, it should be noted, still allow the tissue to maintain a concentration of potassium much above that of the surrounding medium, and to exhibit intracellular potentials (Hillman & McIlwain, 1961; H. H. Hillman, unpublished work).

(iii) On the other hand, the tissues' respiratory response to applied pulses has now been shown to be dependent on the presence of an appreciable concentration of potassium. Similar dependence on sodium salts was demonstrated previously (Gore & McIlwain, 1952). A primary action of the pulses thus appears to be on ion movement rather than on the other metabolic changes mentioned

(these being associated, rather, with processes of recovery). Present investigations have not given information on whether such recovery processes operate primarily on sodium or potassium salts, or on both.

(iv) Within the earliest periods observed in the present study, measurement of ^{42}K showed not only an increased efflux of potassium in response to pulses but also an augmented influx. These findings indicate an initial effect of the pulses to be one of causing not so much a particular movement of potassium as of causing an increase in permeability to potassium. This is the situation also in peripheral nerve.

Speed of potassium movement

(i) Table 6 indicates that the normal stability of tissue potassium involves approximately equal rates of influx and efflux of some 400 μequiv./g./hr.; pulses increased the net loss (and also the fluxes measured by ^{42}K) by some 240 μequiv./g./hr. This increase, caused by application of 100 pulses/sec., amounts to 0·67 μm-equiv./g. of tissue/applied pulse. The total efflux under these conditions is about three times this amount. These values may be compared with that of Hodgkin & Huxley (1947, Table 2) for the isolated axon of *Carcinus maenas*, from which efflux of 2·1 μmoles of potassium/g. of tissue/impulse can be calculated. The present investigation appears likely to give a minimum value only, for stable rather than maximal values have been used in interpreting Figs. 3–5, and no examination has been made of the effect of pulse frequency or duration on potassium movement. The pulse characteristics adopted represent values a little above the minima found necessary for maximal increase in tissue respiration in an earlier study (McIlwain, 1954).

(ii) On cessation of application of pulses, the tissue content of potassium increased at the considerable rate of 550–600 μequiv./g./hr., returning to a stable value within a minute. Increased respiration and glycolysis have been observed to persist after cessation of pulses (McIlwain, 1954; McIlwain & Tresize, 1956); the conditions under which these observations were made were not entirely comparable with those of the present experiments, and suggested persistence of increased respiration and glycolysis for periods of 5–20 sec. rather than 1 min. Assuming, however, that the increase in rate of respiration of 60 μmoles of oxygen/g./hr. and in glycolysis of 100 μmoles of lactate/g./hr. represent the source of energy for processes leading to reassimilation of potassium, provision of $(60 \times 6) + 100$ μmoles of energy-rich phosphate, or 460 μmoles of $\sim$P/g. of tissue/hr., is implied. This gives a ratio of K^+ ions/$\sim$P of 575/460, or 1·25. This is the order of magnitude suggested for comparable processes in other tissues (where the work to be performed is similar, differential ion concentrations being comparable). Hodgkin & Keynes (1954; see also Davies, 1954) suggested 4 sodium atoms to be transported/mole of oxygen consumed, both in *Sepia* axon and in frog muscle, implying a sodium/$\sim$P ratio of 0·67. Similar ratios were found by Caldwell, Hodgkin, Keynes & Shaw (1960). In the erythrocyte, Glynn (1958) considered the probable ratio of sodium and potassium exchanged/$\sim$P to be greater than 1.

The rate of energy consumption in reassimilating potassium to the tissue in the present conditions may be calculated by using the formula

$$-\Delta F = RT \log_n C_1/C_2,$$

as did Krebs *et al.* (1951; though this does not take into account the electrical potential across the membrane) with the values R 1·987 cal./degree and T 310°; but with C_2, the external concentration of potassium salts, as 6 mM, and C_1, the internal concentration, as 100 mM (making allowance for the tissue extracellular space: see above and Pappius & Elliott, 1956*a*, *b*). This gives a value of 1740 cal. required/g. equiv. of potassium assimilated. Assimilation at the rate of 575 g.-equiv./g. of tissue/hr. therefore requires 1 kcal./g./hr. Taking the free energy (F) available from a mole of adenosine triphosphate as 12 kcal., the provision of 460 μmoles of $\sim$P/g. of tissue/hr. is equivalent to 5·5 cal./g./hr. The augmented rate of potassium reassimilation after stimulation thus appears to use at least 18% of the additional energy supply. Scope nevertheless remains for its utilizing the higher proportion suggested above, and also for the concomitant resynthesis of phosphocreatine or performance of other energy-consuming processes.

SUMMARY

1. Tissue from the cerebral cortex of the guinea pig, incubated in glucose media in apparatus permitting the application of electrical pulses and subsequent rapid transference of tissue, resynthesized phosphocreatine and reassimilated potassium salts to a concentration of 63 μequiv./g. wet. wt.

2. Electrical pulses diminished the potassium content at an initial rate of 240 μequiv./g./hr., until after 10 min. a new steady state was reached with about 80% of the original potassium content. No comparable phenomenon occurred with slices of liver. Stopping pulses after 10 min. was followed in cerebral tissues by rapid reassimilation of potassium, the net gain being at about 600 μequiv./g./hr.

3. During preincubation in media containing ^{42}K, the tissue assimilated the isotope, which was

subsequently released to fresh media at rates implying an initial loss of potassium of 400 μequiv./g./hr., increased to 600–750 μequiv./g./hr. by electrical pulses.

4. The rate of entry of ^{42}K to the tissue was also increased by electrical pulses, implying a change of potassium movement from an unstimulated rate of some 330 to one of 645 μequiv./g./hr.

5. When incubation media contained no added oxidizable substrate, less potassium was assimilated to the tissue but it underwent a similar fall with applied pulses. ^{42}K was again taken up by the tissue, but on transference to fresh media loss of the isotope was more rapid than when glucose was present. Pulses increased loss of ^{42}K as with added glucose.

6. In media with glutamate as only oxidizable substrate, slices were normal in potassium content and again lost potassium with pulses. In media with glucose and glutamate, more potassium was associated with a given initial weight of tissue and again pulses diminished potassium content.

7. Slices prepared and incubated in glucose media which lacked potassium salts contained 5–20 μequiv. of potassium/g. of tissue, according to their former position in the brain and their exposure to potassium-free media. In media 1·1 mM in potassium salts, slices contained 15–30 μequiv. of potassium/g.

8. In slices with potassium contents of 5–30 μequiv./g. of tissue, an approximately linear relationship was found between content of potassium and the increase in respiration brought about in the tissue by applied electrical pulses.

9. It is concluded that an early effect of pulses is to increase the permeability of the tissue to potassium salts, permitting the increase in both influx and, preponderantly, efflux of K^+ ions; the increased efflux corresponded to a minimum loss of 0·67 μm-equiv. of potassium/g. of tissue/applied pulse.

10. Computations suggest that some 1·25 μequiv. of potassium is reassimilated/equiv. of energy-rich phosphate made available by the concomitant increase in respiration and glycolysis; the reassimilation may take 18% of this additional energy supply.

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An Attachment of Protamines to Cerebral Tissues, Studied in Relation to Gangliosides, Suramin and Tissue Excitability

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Cerebral tissues incubated in oxygenated glucose media normally respond to electrical pulses by a number of metabolic changes (McIlwain, 1951, 1959*a*); if basic proteins are added to such incubation media, response to pulses is diminished or lost (McIlwain, 1959*b*). Loss of response is accompanied by a change in the tissue gangliosides, of which a greater proportion becomes fixed to the tissue in a form not immediately extractable by chloroform–methanol mixtures (S. Balakrishnan & H. McIlwain, in preparation). Concomitant changes in the fluids of the tissue have also been observed, as is reported below. Small quantities of basic proteins suffice to cause the loss of excitability, for example 0·15 mg. of a protamine sulphate/ml. of incubation medium. In these concentrations protamine can, however, be detected by precipitation reactions before the medium is incubated with cerebral tissues. After incubation, the protamine so detectable in the medium diminished (McIlwain, 1960, 1961).

The simplest interpretation of these phenomena is that the protamines are taken up from the medium by combination with the tissue gangliosides, into complexes not extractable with chloroform–methanol mixtures. Other explanations are not, however, excluded, for gangliosides form soluble complexes with the basic proteins (McIlwain, 1960, 1961). Direct evidence for attachment of basic proteins to tissue slices has therefore been sought by analysis of the incubation medium and of the tissue itself. Preliminary experiments showed that it was possible to determine protamines in such materials by their constituent arginine, as was done by Wolfe & McIlwain (1961), and this has been made the basis of the present study.

Excitability can be restored to tissues which have been exposed to protamines, by subsequent incubation with ganglioside-containing solutions; also, if added together with adequate quantities of gangliosides, protamines are no longer inhibitory. Distribution of protamines between tissue and solution under these conditions also has been examined. Certain other substances act as do gangliosides in restoring tissue excitability, though greater concentrations are required of the other antagonistic agents (McIlwain, 1960, 1961 and unpublished work). One of these substances, the sodium salt of bis-(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate) carbamide, has been included in the present study. It is the antitrypanosomal drug suramin.

EXPERIMENTAL

Tissue metabolism. Guinea-pig brains were obtained and slices of cortex prepared from the cerebral hemispheres, and weighed, as described by Marks & McIlwain (1959). Slices from one animal were used in each of the experiments quoted in the Results section. Tissue weighing 50–70 mg. was incubated at 37·5° in 3·5 ml. of the phosphate medium of Rodnight & McIlwain (1954), oxygenated and with the additions noted in individual experiments. Respiration was followed manometrically in conventional Warburg apparatus, readings being taken at 5 min. intervals. Immediately after incubation, the contents of the main compartment of a vessel were tipped into a shallow dish and the tissue was transferred with a spatula to a second dish of medium and immediately to a tube for hydrolysis. This was done successively and promptly with each vessel. Electrical stimulation employed the silver-grid electrodes of Ayres & McIlwain (1953), to which were applied alternating condenser pulses of 10 v peak potential, 0·4 msec. time constant, at 100/sec.

Arginine assay. Tissues, and fluids after centrifuging for 5 min. at 800*g*, were hydrolysed according to Wolfe & McIlwain (1961) to liberate protein arginine. Incubation fluids (1 ml.) were placed with 1 ml. of 10 N-HCl and 30 μl. of 12·5% (w/v) titanous chloride in 16 mm. × 150 mm. Pyrex tubes. The tubes were sealed with a blowlamp and kept at 110–120° for 4 hr. Tissues were ground in similar tubes with 1 ml. of 10 N-HCl with a glass pestle, which was subsequently washed in with 0·5 ml. of 10 N-HCl and 1·5 ml. of water, 40 μl. of the titanous chloride was added and hydrolysis completed as described above.

After hydrolysis, the contents of the tubes were neutralized (shown by maximal precipitation of titanium hydroxides) with 6 N-NaOH, filtered and diluted for assay. Diluted extracts were found to be stable for 24 hr. at 0°, but for longer storage it was found best to keep the specimens in their sealed tubes before hydrolysis and at 0°. Arginine was determined by Satake & Luck's (1958) method, with internal standards in the range 20–60 μM-arginine. Estimations were carried out in triplicate and a reagent blank was included with each group. Because of

the removal of a small precipitate by centrifuging, not all arginine of the incubation mixtures is accounted for, e.g. in the experiments of Table 1. The centrifuging was carried out in order that tissue arginine could be compared with arginine in solution in the ambient fluid, and not in suspension.

Determination of suramin. In fluids, suramin was determined according to Gage, Rose & Scott (1948), with a Unicam spectrophotometer and measuring E at 505 mμ. Tissues (approx. 60 mg.) were ground in 3 ml. of 0·1 N-NaOH in a test-tube homogenizer and after 15 min. at 0° the mixture was centrifuged (15 min., 800g). To 2 ml. of supernatant were added: 0·5 ml. of 0·1 M-KH_2PO_4 brought to pH 7·4 by NaOH; 20μl. of Universal indicator (British Drug Houses Ltd.); 0·1 N-HCl to neutrality. Of the neutralized extract, 0·2 ml. was taken for analysis as described above. The tissue extracts gave an apparent suramin content of 1·0–1·5 mg./g. of tissue when no suramin had been added; this blank was not subtracted from the values quoted. Recovery of suramin added to the alkaline tissue extracts was 92–95%.

Materials. L-Arginine monohydrochloride was from L. Light and Co. Ltd. Two specimens of protamine sulphate were used: the analysed specimen of the clupein salt, from herring, described by McIlwain (1960, 1961) and found by the above-described assay to yield 0·41 g. of arginine/g.; salmine sulphate (British Drug Houses Ltd.), which yielded 0·43 g. of arginine/g. Two ganglioside preparations were made from ox cerebral cortex largely by Folch, Lees & Sloane-Stanley's (1957) method, as described by McIlwain (1960, 1961). One specimen contained 26·5% of *N*-acetylneuraminic acid when assayed according to Long & Staples (1959; for additional details see McIlwain, 1960, 1961); a second, for which we are indebted to Dr S. Balakrishnan, contained 28%; these materials are mixtures of more than one molecular species (Meltzer, 1958; Folch & Lees, 1959; Klenk & Gielen, 1960; D. A. Booth, personal communication). Suramin was obtained from Imperial Chemical Industries Ltd. (Pharmaceuticals Division) and from Farbenfabriken Bayer A.-G. We are indebted to Dr F. L. Rose of Imperial Chemical Industries Ltd. (Pharmaceuticals Division) for 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride (I.C.I. 5302; SQ 24 of Browning, Cohen, Ellingworth & Gulbranson, 1926) and for indicating its use in determining suramin. Other materials obtained were of A.R. grade.

RESULTS

Arginine content of cerebral tissues

Slices of guinea-pig cerebral cortex, cut and floated in phosphate medium and drained and weighed but not incubated, contained 7·8 ± 0·73 mg. of arginine/g. of tissue from one animal, and from another, 7·7 ± 0·54 (S.D.; six and five slices respectively). After incubation for 60 min. at 37·5° in oxygenated phosphate medium, 6·16 ± 1·35 mg. of arginine/g. of tissue was found in 15 tissues from eight guinea pigs; the incubation fluids then contained arginine equivalent to 1·19 ± 0·47 (15) mg./g. of tissue.

These values indicated the feasibility of following the fate of added protamine by arginine assays, for protamine sulphate diminishes tissue excitability when added at 0·05–0·25 mg./ml. of incubation fluid, with a tissue/fluid ratio of 20 mg./ml. (McIlwain, 1960, 1961). This involves addition of 2·5–12·5 mg. of protamine sulphate or 1·1–5·5 mg. of arginine/g. of tissue. Protein constitutes about 10% of the brain, and mixed cerebral proteins contain 5–5·4% of arginine (Block, Jervis, Bolling & Webb, 1940), indicating the greater part of the arginine determined above to be derived from protein; the free arginine is small (Blass, 1960).

Table 1. *Protamine uptake by guinea-pig cerebral cortex*

Protamine was added to the phosphate medium (3·5 ml.) before the tissue was placed in it. Respiration was measured at 37·5° for 60 min. (giving rates of 58–65 μmoles of O_2/g. of tissue/hr.), after which arginine was determined. For other details, see the Experimental section.

		Tissue		Fluid	
Expt. no.	Substance added (mg./ml.)	Initial wt. (mg.)	Final arginine (mg./g.)	Arginine (μg./vessel)	Arginine (mg./g. of tissue)
1	None	50	6·2	53	1·1
		70	5·4	99	1·4
	Clupein SO_4 (0·06)	60	10·3	143	2·4
		70	7·2	236	3·4
	Clupein SO_4 (0·1)	65	9·6	124	1·9
		60	12·5	178	3·0
2	None	—	5·9 ± 0·8 (4)	—	0·9
	Clupein SO_4 (0·1)	63	7·7	89	1·4
		54	7·5	320	5·9
3	None	63	6·6	44	0·7
	Salmine SO_4 (0·1)	56	8·8	107	1·9
		43	8·4	125	2·9
	Salmine SO_4 (0·2)	59	10·5	258	4·4
		49	10·4	254	5·2

Table 2. *Course of protamine uptake*

Tissues were incubated for 10 min. in protamine-free media; clupein sulphate (0·214 mg./ml.) was then added and, after the intervals indicated, tissues were removed for analysis. Each result is the mean of duplicate determinations on each of two slices; other details are as given for Table 1.

Expt. no.	Arginine of the protamine SO_4 added (μg./ml. of medium)	Time of incubation with protamine (min.)	Arginine content	
			Tissue (mg./g.)	Fluid (mg./g. of tissue present)
1	0	10	5·0	—
	110	10	5·7	—
	0	30	5·0	—
	110	30	6·7	—
2	0	30	3·8	1·4
	108	30	7·7	8·5
	0	60	3·9	2·6
	108	60	7·8	8·1

Table 3. *Protamine uptake in the presence of gangliosides*

Tissues were incubated for 1 hr. in 3·5 ml. of phosphate medium containing from the start the protamine with or without gangliosides. Each value for tissue content is the mean of four determinations: two estimations on each of duplicate slices. Each value for fluid arginine is the mean of two estimations. Other details are as given for Table 1.

Expt. no.	Substances added (mg./3·5 ml.)	Arginine content	
		Tissue (mg./g.)	Fluid (mg./g. of tissue)
1	None	8·2	1·8
	Clupein SO_4 (0·75)	10·4	7·2
	Clupein SO_4 (0·75); ganglioside (2)	6·7	8·8
2	None	5·8	0·6
	Clupein SO_4 (0·75)	9·8	5·4
	Clupein SO_4 (0·75); ganglioside (1)	7·6	6·1
3	None	6·3	—
	Ganglioside (1)	5·4	—
	Ganglioside (2)	4·5	—

Table 4. *Gangliosides added to protamine-treated slices*

The design of Expts. 1 and 2 was that of Table 3 of McIlwain (1961). In the first 15–20 min. period, respiratory rates were 58–65 μmoles of O_2/g. of tissue/hr.; the media were then replaced by those of the second period (40 min.) and rates were 49–60 μmoles of O_2/g./hr. In Expt. 3, after 30 min. incubation, constituting the first period, ganglioside was added from the side arms of the vessels indicated and an equivalent volume of medium was added to the others; incubation was continued for a further 30 min. Each result is a mean of four determinations: two estimations on each of two slices.

Expt. no.	Additional substances present (mg./3·5 ml.)		Arginine of tissue (mg./g.)
	First period	Second period	
1	None	None	4·5
	Clupein SO_4 (0·75)	None	6·4
	Clupein SO_4 (0·75)	Ganglioside (2)	6·7
2	None	None	4·2
	Clupein SO_4 (0·75)	None	5·5
	Clupein SO_4 (0·75)	Ganglioside (2)	5·3
3	None	None	5·5
	Clupein SO_4 (0·75)	Clupein SO_4 (0·75)	7·8
	Clupein SO_4 (0·75)	Clupein SO_4 (0·75); ganglioside (2)	9·1

Incubation with protamine; effect of gangliosides

Slices of cerebral cortex incubated in media containing 0·06–0·2 mg. of protamine sulphate/ml. increased in arginine content (Table 1). The increase took place with both protamine specimens examined; uptake was marked between 10 and 30 min. of incubation but had increased little at 60 min., although appreciable protamine remained in solution (Table 2).

The concentrations of gangliosides found (McIlwain, 1960, 1961) to interact with the concentrations of protamine now being studied were of 0·1–0·5 mg./ml. The effect of such additions has been investigated in three ways. In the first (Table 3) gangliosides were mixed with protamine before the tissues were added for incubation. After incubation in such mixtures, the usual uptake of protamine was found to have been partly or completely blocked, according to the amount of ganglioside added. Possibly the higher concentration of ganglioside withdrew arginine-containing material from the tissue itself. This was the case when tissues were incubated with gangliosides only. In the second type of experiment (Table 4, Expts. 1 and 2) tissues were initially incubated in protamine-containing solutions for 15–20 min. The media were then removed and replaced either by media without addition or by media with gangliosides. In this situation, gangliosides can restore tissue excitability (McIlwain, 1960, 1961). They did not, however, diminish the tissue content of protamine. In a further experiment (Expt. 3, Table 4) gangliosides were added during incubation and without prior removal of the protamine-containing solution. The results suggested that addition of the gangliosides promoted a further binding of protamine to the tissue.

Protamine and suramin

Suramin at 0·25–2 mg./ml. of incubation fluid partly restores excitability to tissues previously exposed to protamine, and its effect on tissue protamine has accordingly been examined (Table 5). Analyses for tissue arginine carried out after incubation in protamine–suramin mixtures (Expt. 1) showed that suramin could diminish the uptake of protamine; it also formed a precipitate with protamine. However, protamine, once attached to the tissue, was not removed by subsequent incubation with suramin (Expts. 2–4, Table 5). The findings with suramin thus paralleled those with gangliosides.

The suramin content of tissues treated in this fashion has also been estimated. Much suramin was found to be taken up by the tissue, whether or not it had first been incubated with protamine (Table 6). Although the quantity of suramin and the period of incubation with it were varied in the different experiments, no evidence was found for protamine causing a major increase in the uptake of suramin by the tissue. The only indication of such uptake is in Expt. 3, Table 6, and is not large.

Other observations

In Tables 1–6 tissue constituents have been expressed in terms of the weight of the slices determined at the commencement of the experiments immediately before incubation. Such slices normally increase in weight during incubation by

Table 5. *Suramin and tissue protamine*

Expt. 1 was performed as described in Table 3, ganglioside being replaced by suramin. Expts. 2–4 were performed as described in Table 4, Expts. 1 and 2, ganglioside being replaced by suramin.

	Substances added (mg./3·5 ml.)		
Expt. no.	First period	Second period (suramin)	Arginine content (mg./g. of tissue)
1	None	—	5·0
	Clupein SO_4 (0·75)	—	7·0
	Clupein SO_4 (0·75); suramin (0·75)	—	5·3
2	None	0	5·1
	Clupein (0·75)	0	7·7
	Clupein (0·75)	0·75	6·8
3	None	0	4·7
	Clupein (0·75)	0	6·0
	Clupein (0·75)	3·5	6·1
4	None	0	4·3
	Clupein (0·75)	0	5·2
	Clupein (0·75)	7·0	5·6

Table 6. *Attachment of suramin to cerebral tissue*

Expts. 1 and 2 were carried out as described in Table 4, Expts. 1 and 2. In Expt. 3, suramin was placed in the side arms of the Warburg vessels and tipped in after preincubation for 20 min. After incubating for a further 5 min., the vessels were removed and suramin was estimated as described in the Experimental section. The tissues give an apparent suramin value when none is added.

	Substance added (mg./3·5 ml.)		Suramin or apparent suramin	
Expt. no.	First period (clupein SO_4)	Second period (suramin)	Tissue (mg./g.)	Fluid (mg./3·5 ml.)
1	0	0	0·7	0·014
	0	3·5	4·1	3·5
	0·75	3·5	3·9	3·4
2	0	0	1·5	0·02
	0	0·75	3·6	0·70
	0·75	0·75	3·3	0·66
3	0	0	1·48	0·016
	0	0·75	1·53	0·73
	0·75	0·75	1·86	0·56

absorption of fluid (Pappius & Elliott, 1956; Leaf, 1956; Cummins & McIlwain, 1961). Protamine in the concentrations employed in the present experiments has now been observed to diminish such uptake of fluid (Fig. 1). It also causes a small change in the appearance of the tissue, which at the end of incubation in protamine-containing solutions is slightly more whitish and opaque when compared with slices incubated without protamine; these retain to a greater extent their original pale, partly translucent, grey appearance. The change with protamine is seen best when slices with and without additions are floated together in a shallow, black-glazed dish; it becomes more obvious when larger quantities of protamine are added. Moreover, the changes both in appearance and in uptake of fluid are in part antagonized by added gangliosides (Fig. 1).

DISCUSSION

The action of protamine on the excitability of cerebral tissues (McIlwain, 1959*b*, 1960, 1961) has now been shown to be accompanied by the attachment of protamine to the tissue. Results of Table 1 indicate the tissue to acquire rather more than half the quantity of protamine added. As in these experiments tissues of 60 mg. were incubated in 3·5 ml. of fluid, a 60-fold concentration in the tissue is implied. This computation is approximate only, it being supposed that the protamine does not affect the distribution of other arginine-containing substances.

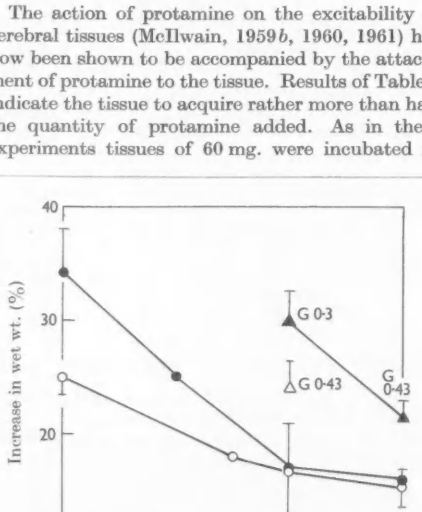

Fig. 1. Change in weight of guinea-pig cerebral cortex after incubation for 80 min. Clupein sulphate and gangliosides (G, mg./ml.) were present in the quantities indicated, in glycylglycine–glucose medium (McIlwain, 1959*b*), throughout the experiments. Electrical pulses, when indicated, were applied for the last 35–40 min. of the experiment. Tissue with protamine only: no pulses, ○; with pulses, ●. Tissue with protamine and gangliosides: no pulses, △; with pulses, ▲. Vertical lines extend from points to distances equivalent to the S.D. (four to six values); other points are means of two values.

A migration of the basic proteins native to cerebral tissues, which also leads to loss of tissue excitability, was previously concluded (McIlwain, 1959*b*; Wolfe & McIlwain, 1961; Wolfe, 1961) to involve attachment of the proteins to membrane structures. Evidence for this was given by analysis of fractions of ground cerebral tissues after differential centrifuging. As it is known that excitation in isolated mammalian cerebral tissues, as in other systems, is associated with ion movements (Cummins & McIlwain, 1961) across membranes which are normally polarized (Li & McIlwain 1957), attachment of the inhibitory proteins at the 'active' cell membrane involved in ion movement offers a reasonably direct picture of the actions of the proteins on tissue excitability. Basic polypeptides have been concluded to agglutinate erythrocyte suspensions by drawing together adjacent cells through attachment of the peptide molecules between the membranes of pairs of cells (Katchalsky, Danon, Nevo & de Vries, 1959). It appears feasible that such action on the part of protamine could account for the changes in appearance and in water content that it causes in the tissue. Surface membranes of cell processes in the rat cerebral cortex have been observed to be some 200–400 Å apart, and tubules of the endoplasmic reticulum to be some 200 Å in diameter (Gray, 1958, 1959).

The site of attachment of protamine is to be characterized chemically as well as in terms of cell structure; the previous suggestion that attachment was to ganglioside-containing materials (McIlwain, 1960, 1961) was based on extraction and fractionation of naturally occurring materials in search of compounds able to antagonize the inhibitory effects of the basic proteins. Antagonism by gangliosides is now seen to extend to other aspects of the interaction between protamine and tissue: to the attachment of protamine to tissue slices (Table 3) and to the effect of protamine on tissue fluids (Fig. 1). Certain related findings were, however, unexpected: when gangliosides were added to cerebral tissues which had lost excitability through incubation with protamine, excitability was largely restored (McIlwain, 1960, 1961) but protamine remained attached to the tissue (Table 3, this paper). There are even suggestions that gangliosides further increase the binding of protamine under these conditions.

Tissue gangliosides are being made the subject of an independent study (S. Balakrishnan & H. McIlwain, in preparation); the results of the parallel experiments with suramin may be dis-

cussed here. Suramin readily combines with many tissues and proteins of the body (Boursnell & Wormall, 1939; Spinks, 1948). Under conditions in which it increases the excitability of protamine-treated tissue, much suramin is taken up by the slices (Table 6). As both protamine and suramin are highly water-soluble substances, their attachment to the tissue is a very real phenomenon. The values of Table 6 imply a four- to ten-fold concentration of suramin in favour of the tissue. The attachment of suramin is largely independent of added protamine, but may be compared in quantity with the protamine which becomes attached under similar conditions. From Tables 3–5 incubation with 0·75 mg. of protamine/vessel is seen to lead to uptake of approx. 2·5 mg. of arginine/g. of tissue, or 6·1 mg. of protamine/g. This is approx. 1·2 μ-mole/g., and 7·2 or 22 μequiv./g., according to the basicity considered relevant (for sources of data see McIlwain, 1960, 1961). On incubation with 0·75 and 3·5 mg. of suramin/vessel, tissues take up 2 and 3·3 mg. of suramin/g. (Table 6). With a mol.wt. of 1466 these quantities correspond to 1·4 and 2·3 μmoles/g., or 8·2 and 13·5 μequiv./g. Computed in these different ways therefore similar quantities of acidic and basic substances are being attached to the tissue during these experiments. Suramin appears unlikely to simulate gangliosides in fashions other than its being a multivalent anion of large molecular weight, and the present experiments therefore contribute to the picture (McIlwain 1960, 1961) of negatively charged surface structures being required for the ion movements associated with tissue excitability.

SUMMARY

1. Protamine added to media in which cerebral cortical tissue is incubated is taken up by the tissue, which loses its metabolic response to electrical pulses. Uptake is small in 10 min. but nearly maximal in 30 min.

2. Uptake of protamine is diminished by concentrations of gangliosides which diminish the action of protamine on tissue excitability. Protamine already attached to the tissue is not, however, removed by subsequent incubation in ganglioside-containing solutions.

3. Protamine diminishes the uptake of fluid that normally occurs on incubating cerebral tissues *in vitro*, both with and without application of electrical pulses. Gangliosides oppose protamine in this respect also.

4. Suramin, which also diminishes the action of protamine, again prevents the binding of protamine by cerebral tissues but does not remove protamine already attached.

5. Suramin added to incubating fluids itself becomes attached to cerebral tissues; the quantity bound is independent of previous exposure of the tissue to protamine, but is similar to that of the protamine whose action it opposes.

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The Distribution of Gangliosides in Subcellular Fractions of Guinea-Pig Cerebral Cortex

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Gangliosides are acidic, acid-sensitive, non-dialysable, water-soluble lipids containing *N*-acetylneuraminic acid, hexosamine, hexoses and ceramides which in the brain occur mainly in grey matter. The aim of the present investigation was to study the localization of gangliosides in the cerebral cortex by following their distribution in subcellular fractions, neuraminic acid content being used as marker. This investigation followed from the findings of McIlwain (1960*a*, *b*, 1961) that gangliosides from ox brain, mucopolysaccharides and mucoproteins containing neuraminic acid restore the respiratory response to electrical stimulation in cerebral-cortex slices in which this response had been abolished by cold pretreatment of the tissue or by protamines, histones or synthetic poly-L-lysine. The lack of response to electrical pulses in slices pretreated in cold media appears to be due to a migration of the tissue's histone from the nucleus and its combination with acidic, extranuclear sites (Wolfe & McIlwain, 1961). McIlwain (1960*a*, 1961) suggested that the inhibitory action of basic proteins is due to combination with tissue gangliosides which may be involved normally in the processes of excitation or recovery. These results were discussed with Dr W. E. van Heyningen, who had tentatively identified gangliosides as receptors of tetanus toxin in neural tissues (van Heyningen, 1959).

A short account of this work has already appeared (Wolfe, 1960).

EXPERIMENTAL

Preparation of homogenates

Guinea pigs were stunned by a blow on the neck, the throat was cut for bleeding to death and the brain removed. The cerebral hemispheres were removed from the brain stem; by opening the ventricles and by dissection with a blunt instrument the layers of subcortical white matter were removed. The cerebral-cortical tissue was then weighed on a torsion balance and placed in ice-cold 0·32M-sucrose adjusted to pH 7·0 (with NaOH). A medium/tissue (v/w) ratio of 10 was used. Homogenization was carried out in a precooled, model 10 Emanuel–Chaikoff hydraulic tissue homogenizer (Microchemical Specialties Co.) with an orifice of 25μ after preliminary brief dispersion in a glass homogenizer with a polythene plunger.

Preparation of subcellular fractions

Method 1: *differential centrifuging.* The fractionation of the 0·32M-sucrose homogenates was carried out in a no. 40 rotor of a Spinco model L preparative ultracentrifuge. All operations were conducted at 0–4° and the separated fractions were examined with a phase-contrast microscope. The following fractions were prepared: (1) The residue sedimenting after 10 min. at 800 *g* (R1) consisting mainly of nuclei, blood vessels and myelin fragments. (2) The residue sedimenting after 10 min. at 5000 *g* (R2) consisting mainly of mitochondria but also containing small glial nuclei and whorls of myelin. (3) The residue sedimenting after 10 min. at 20 000 *g* (R3) consisting of small mitochondria and large microsomal particles. (4) The residue sedimenting after 95 min. at 105 400 *g* (R4) consisting of the microsomal and post-microsomal particles. When examined under the high power of the phase-contrast microscope this fraction appears as a shimmering mass of indistinct particles. (5) The supernatant (S1). Fractions R1 and R2 were washed twice and R3 once by resuspension in 10 vol. of 0·32M-sucrose and centrifuging. The washings were added to the next fraction. After each centrifuging the supernatant fractions were removed with a Pasteur pipette. Particular care was necessary in removing the supernatant (S1) from the microsomal fraction (R4) to avoid disturbing the smallest particles on the surface of the pellet.

Method 2: *differential centrifuging and subfractionation in density gradients.* The separation of the primary subcellular fractions and further subfractionation of the 'crude mitochondrial' fraction was by equilibrium centrifuging in sucrose density gradients. The following fractions were prepared: (1) The residue sedimenting after 10 min. at 800 *g* (P1). (2) The residue sedimenting after 20 min. at 22 500 *g* (P2). (3) The residue sedimenting after 95 min. at 105 400 *g* (P3). (4) The supernatant (S2).

The P2 fraction was separated into subfractions by layering the pellet resuspended in 10 ml. of 0·32M-sucrose over a density gradient consisting of 10 ml. of 0·8M-sucrose and 10 ml. of 1·2M-sucrose and centrifuging in the SW 25·1 rotor of the Spinco ultracentrifuge, at 53 500 *g* for 140 min. Three distinct layers were apparent after centrifuging. (1) A layer (P2A) of white material between the 0·32 and 0·8M-sucrose layers. (2) A layer (P2B) of greyish-white material between the 0·8 and 1·2M-sucrose layers. (3) A tan-coloured residue (P2C) beneath the 1·2M-sucrose layer. The layers were separated by means of a Pasteur

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pipette with the tip bent at right angles to the body of the pipette. The separated layers were brought to approx. 0·3 M-sucrose and centrifuged at 105 400 *g* for 20 min.

Determination of gangliosides

Extraction. The particulate fractions prepared as described above were extracted with cold (4–5°) $CHCl_3$–methanol (2:1, v/v) and subjected to solvent partition as described by Folch, Lees & Sloane-Stanley (1957), the choice of some conditions being those of Long & Staples (1959). The procedure used is outlined in Fig. 1. The supernatant fractions at stage 3 were evaporated to dryness at 60° in a rotary evaporator and the residue was extracted with $CHCl_3$–methanol. Most of the sucrose in the particulate fractions remained in the residue after the second extraction with $CHCl_3$–methanol. The remainder of the sucrose was removed by dialysis. Dialysis was carried out for 16 hr. in 0·25 in. diam. Visking tubing (Viscose Development Co., London) in cylindrical jars (approx. 17 l. capacity) with two changes of water. The water was agitated with a Vibromix stirrer (Shandon Scientific Co. Ltd., London). After dialysis, the sacs were emptied and rinsed and the contents made to a standard volume.

Estimation of neuraminic acid. Measured quantities of the dialysed extracts of the subcellular fractions were evaporated to dryness and determinations of the neuraminic acid content were carried out with Bial's orcinol–$FeCl_3$–HCl reagent according to the procedure of Böhm, Dauber & Baumeister (1954) as modified by Long & Staples (1959). Synthetic *N*-acetylneuraminic acid was used as standard.

Other procedures and materials

Nitrogen in the various subcellular fractions was estimated by the usual micro-Kjeldahl procedure with selenium oxide–$CuSO_4$ mixture as catalyst.

Ultrasonic disruption. In experiments using ultrasonic-disruption procedures a Mullard–MSE ultrasonic disintegrator (60 w, 20 kcyc./sec.) was used. (We are indebted to Professor C. H. Gray for the use of this apparatus.) The suspensions were placed in a thick-walled glass tube supported in an ice bath. The stainless-steel stub, 19 mm. diam., was immersed 4 mm. below the surface.

Materials. All chemicals and solvents used were of A.R. grade. Protamine (clupein sulphate) and lysozyme chloride were obtained from L. Light and Co. Ltd. Calf-thymus histone was prepared according to the procedure of Luck, Rasmussen, Satake & Tsvetikov (1958). The following materials were gifts: a preparation of ox-brain ganglioside, *N*-acetylneuraminic acid content 26·5%, from Professor H. McIlwain; synthetic *N*-acetylneuraminic acid from Miss Patricia Carroll; egg lysolecithin from Professor R. H. S. Thompson.

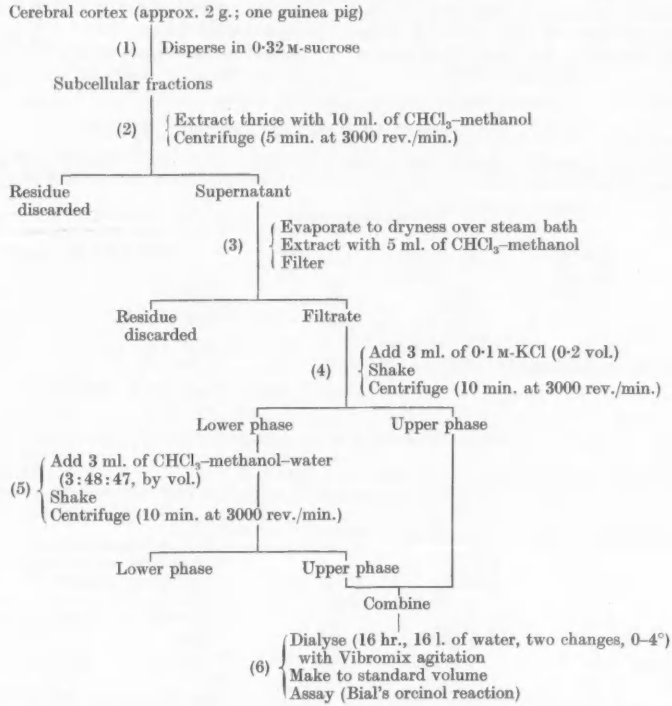
Cerebral cortex (approx. 2 g.; one guinea pig)
(1) Disperse in 0·32 M-sucrose
Subcellular fractions
(2) Extract thrice with 10 ml. of CHCl3–methanol
Centrifuge (5 min. at 3000 rev./min.)
Residue discarded
Supernatant
(3) Evaporate to dryness over steam bath
Extract with 5 ml. of CHCl3–methanol
Filter
Residue discarded
Filtrate
(4) Add 3 ml. of 0·1 M-KCl (0·2 vol.)
Shake
Centrifuge (10 min. at 3000 rev./min.)
Lower phase
Upper phase
(5) Add 3 ml. of CHCl3–methanol–water (3:48:47, by vol.)
Shake
Centrifuge (10 min. at 3000 rev./min.)
Lower phase
Upper phase
Combine
(6) Dialyse (16 hr., 16 l. of water, two changes, 0–4°) with Vibromix agitation
Make to standard volume
Assay (Bial's orcinol reaction)

Fig. 1. Procedure for the extraction of gangliosides from subcellular fractions.

RESULTS

Occurrence of gangliosides in particulate form

When gangliosides were extracted from the particulate and supernatant fractions obtained after centrifuging homogenates of guinea-pig cerebral cortex at 105 400*g* for 30 min., 22 % of the gangliosides occurred in the supernatant fraction. However, if the time of centrifuging was increased to 95 min. (i.e. an integrated field of about 10 000 000*g*-min.) no gangliosides were obtained from the supernatant fractions (Table 1). Therefore under these latter conditions gangliosides apparently occur entirely in particulate fractions. From the results shown in Table 1 it is concluded that some gangliosides occur bound to the very smallest, post-microsomal fragments of cerebral-cortex homogenates. Particular care was required in removing the supernatant fractions from the pellets so that the delicate uppermost material was not disturbed. On occasions when this was disturbed accidentically, gangliosides were recovered from the supernatant fraction.

The values obtained for the total ganglioside content of homogenates are in good agreement with those obtained by Long & Staples (1959) for the grey matter of the rat and by Dr S. Balakrishnan in this laboratory for the grey matter of the guinea pig.

Recovery of added gangliosides. From the results shown in Table 2 it is clear that if an aqueous solution of ox-brain ganglioside is added to homogenates 94 % of it is recovered in the total particulate fraction. When the nuclear fraction was analysed 87 % of the added gangliosides were recovered. From these results it appears that soluble gangliosides added to homogenates of cerebral cortex form an insoluble complex with some component of the nuclear fraction. Evidence presented below suggests that these components are the nucleohistones.

Distribution of gangliosides in subcellular fractions

Fractions prepared by differential centrifuging only. The ganglioside contents of subcellular fractions prepared by Method 1 (see the Methods section) are shown in Table 3. It is shown that the microsomal fraction (R 4) contains the greatest amount of gangliosides and the mitochondrial fraction (R 2) the least. Unwashed nuclear fractions were found to contain up to 110 μg. of *N*-acetylneuraminic acid/g. of fresh tissue in the extracts. However, this could be decreased by approximately 50 % with two washings with 0·32 M-sucrose. Washing up to four times did not further lower the ganglioside content.

Subcellular fractions were prepared by Method 2 (see the Methods section) without further sub-

Table 1. *Occurrence of gangliosides in particulate fractions*

Procedures for the extraction of gangliosides and the determination of *N*-acetylneuraminic acid are described in the Methods section. The values are converted into μmoles by dividing by 309 (mol.wt. of *N*-acetylneuraminic acid).

Fraction	Centrifugal force: *g*	Centrifugal force: min.	*N*-Acetylneuraminic acid (μg./g. fresh wt. of tissue) (mean ± S.E.M.)
Residue	105 400	30	312 ± 16 (3)
Supernatant	—	—	88 ± 9 (3)
		Total	400 ± 11 (1·28 ± 0·04 μmoles)
Residue	105 400	95	476 ± 87 (8)
Supernatant	—	—	0 (8)
		Total	476 ± 87 (1·54 ± 0·28 μmoles)

Table 2. *Recovery of gangliosides added to homogenates of guinea-pig cerebral cortex*

The 0·32 M-sucrose homogenates were divided into two equal portions and to one portion 400 μg. of ox-brain gangliosides (containing 106 μg. of *N*-acetylneuraminic acid) in 1 ml. of 0·32 M-sucrose was added and to the other portion 1 ml. of 0·32 M-sucrose. The portions were then centrifuged at 105 400*g* for 95 min. or the nuclear fraction was separated as outlined in the Methods section.

Fraction	Centrifugal force: *g* (av.)	Centrifugal force: min.	*N*-Acetylneuraminic acid (μg./g. fresh wt. of tissue) (mean ± S.E.M.): Gangliosides added	*N*-Acetylneuraminic acid (μg./g. fresh wt. of tissue) (mean ± S.E.M.): No gangliosides added	Mean recovery (%)
Total particulate	105 400	95	527 ± 48 (3)	427 ± 50 (3)	94
Supernatant	—	—	0	0	—
Nuclear	800	10	157 ± 8 (3)	65 ± 10 (3)	87
Ganglioside only in 0·32 M-sucrose	105 400	95	None sedimented		

fractionation of the P2 fraction in a density gradient. The ganglioside content of these fractions is shown in Table 4. It is seen that the P2 fraction contains the greatest amount of gangliosides. Thus an increase in the centrifugal force from 200 000*g*-min. used in the separation of fraction R3 (Table 3) to 450 000*g*-min. used in the separation of fraction P2 (Table 4) results in the sedimenting of particles that contain considerable quantities of gangliosides.

Fractions prepared by differential centrifuging and subfractionation in a density gradient. Subcellular fractions were prepared by Method 2 (see the Methods section) and the P2 fraction was further subfractionated in a sucrose density gradient. The gangliosides present in these fractions are shown in Table 5. The P2 fraction contained the greatest amount of gangliosides. Of the subfractions the middle layer (P2B), consisting of particles intermediate in density between 0·8 and 1·2 M-sucrose, contained 56·5% of the gangliosides of the P2 fraction.

Although the P2B fraction contains the greatest amount of gangliosides expressed as μg. of *N*-acetylneuraminic acid/g. fresh wt. of tissue, if the results are expressed in μg. of *N*-acetylneuraminic acid/mg. of nitrogen the microsomal fraction, P3, contains the highest concentration of gangliosides.

Table 3. *Distribution of gangliosides in subcellular fractions of guinea-pig cerebral cortex*

Fractions were prepared by Method 1 as outlined in the Methods section. The ganglioside contents are expressed as μg. of *N*-acetylneuraminic acid in the dialysed, aqueous-partitioned $CHCl_3$–methanol extracts of the fractions.

Fraction	Centrifugal force		*N*-Acetylneuraminic acid	
	g	min.	(μg./g. fresh wt. of tissue)	% of total
R1	800	10	52	11·4
R2	5 000	10	16	3·5
R3	20 000	10	82	17·8
R4	105 400	95	309	67·3
S1	—	—	0	0
		Total	459 (1·48 μmoles)	

Table 4. *Distribution of gangliosides in subcellular fractions of guinea-pig cerebral cortex*

The primary fractions were prepared by Method 2 as outlined in the Methods section. Other details were as described in Fig. 3.

Fraction	Centrifugal force		*N*-Acetylneuraminic acid	
	g	min.	(μg./g. fresh wt. of tissue)	% of total
P1	800	10	40	9·0
P2	22 500	20	268	60·9
P3	105 400	95	139	31·1
S2	—	—	0	0
		Total	447 (1·45 μmoles)	

Table 5. *Distribution of gangliosides in subcellular fractions of guinea-pig cerebral cortex*

The primary fractions were prepared by Method 2 as outlined in the Methods section. The P2 fraction was layered over a sucrose density gradient. The P2A fraction contains particles in the upper layer, the P2B contains particles from the middle layer and the P2C contains particles passing through the 1·2 M-sucrose layer.

Fraction	*N*-Acetylneuraminic acid		Nitrogen (mg./g. fresh wt. of tissue)	*N*-Acetyl-neuraminic acid (μg./mg. of nitrogen)
	(μg./g. fresh wt. of tissue)	% of total		
P1	53	9·2	3·0	17
P2A	140	24·4	2·5	56
P2B	231	40·2	4·7	50
P2C	38	6·6	1·8	21
P3	112	19·6	1·3	86
S2	0	0	4·8	0
Total	574 (1·87 μmoles)		18·1	—

Distribution of gangliosides in fractions after ultrasonic disruption

Ultrasonic treatment (for 10 min.) of the suspended particles remaining after the removal of the nuclear fraction caused a shift in the *N*-acetylneuraminic acid-containing material from the P2 to the P3 fractions with a small amount appearing in the supernatant fraction (Tables 6*A* and *B*). Therefore ultrasonic treatment disrupts particles normally sedimenting in the P2 fraction so that they now sediment in the microsomal fraction P3. It does not solubilize gangliosides to any appreciable extent. Considerable solubilization of other constituents does occur, as shown by the increase in nitrogen content of the supernatant fraction. These results indicate that the ganglioside-containing fragments in the P2 fraction, of which the greater proportion are found in the P2B layer (Table 5), are further broken by ultrasonic treatment into smaller fragments which are, however, still sedimentable by centrifuging.

Ultrasonic treatment of homogenates in which the nuclear fraction had not been removed considerably lowered the amounts of gangliosides obtained from the total particulate fraction (Table 6*C*). Ultrasonic irradiation causes the disruption of nuclei and leads to the degradation of nucleoproteins (see Medawar & Zubay, 1959). It is possible that the basic substances released from nuclei after ultrasonic irradiation have combined with the gangliosides associated with other subcellular particles in the homogenate and prevented their extraction by chloroform–methanol (S. Balakrishnan & H. McIlwain, in preparation).

Effect of various treatments of the homogenate on the extraction of gangliosides

Calcium chloride. The addition of calcium chloride (5 mM) to homogenates, followed by their separation into subcellular fractions, caused most of the tissue gangliosides to sediment with the nuclear fraction (P1). This is most likely to be due to clumping and agglutination of cytoplasmic particulates in the presence of bivalent ions.

Incubation and dialysis. Incubation of 0·32M-sucrose homogenates of cerebral cortex in a stoppered vessel for 90 min. at 37·5° and then separation of the homogenate into particulate and supernatant fractions caused a 50 % loss of gangliosides. Dialysis of the homogenate against 16 l. of distilled water for 4 hr. caused an even greater loss of gangliosides (Table 7). Prolonged dialysis for 16 hr. resulted in complete loss of neuraminic acid-containing materials from both particulate and supernatant fractions.

Lysozyme and lysolecithin. Incubation of homogenates with 2 mg. of lysozyme chloride for 30 min. at 37·5° caused no loss or change in the distribution of gangliosides. However, incubation with high concentrations of the cytolytic reagent, egg lysolecithin, caused a considerable loss of the extractable gangliosides from the total particulate fraction without a corresponding increase in the supernatant fraction (Table 7).

Trypsin. Digestion of homogenates with 2 mg. of crystalline trypsin had little effect on the content or distribution of gangliosides (Table 7).

Sodium deoxycholate. Sodium deoxycholate, 0·5 %, in 0·01M-phosphate buffer, pH 7·4, lowered

Table 6. *Distribution of gangliosides in subcellular fractions of guinea-pig cerebral cortex after ultrasonic irradiation*

The fractions were prepared by Method 2 as outlined in the Methods section. (*A*) Ultrasonic treatment (for 10 min.) of the homogenate after the separation of the nuclear fraction. (*B*) No ultrasonic treatment. (*C*) Ultrasonic treatment of the homogenate, nuclei were not removed and the particulate and supernatant fractions were then separated by centrifuging at 105 400*g* for 95 min.

	Fraction	Nitrogen (mg./g. fresh wt. of tissue)	*N*-Acetylneuraminic acid (μg./g. fresh wt. of tissue)
(*A*)	P1	2·4	39
	P2	3·9	88
	P3	4·8	239
	S2	6·4	14
	Total	17·5	380 (1·23 μmoles)
(*B*)	P1	2·4	39
	P2	9·7	230
	P3	1·6	94
	S2	4·2	0
	Total	17·9	363 (1·18 μmoles)
(*C*)	Total particulate	—	106
	S2	—	0
		Total	106 (0·34 μmole)

Table 7. *Gangliosides in particulate and supernatant fractions after various treatments*

In these experiments 10 ml. of 0·32 M-sucrose homogenate of guinea-pig cerebral cortex containing 1 g. of tissue was subjected to a variety of treatments and separated into a total particulate and supernatant fraction by centrifuging at 105 400*g* for 95 min. The gangliosides were extracted and determined as outlined in the Methods section.

Treatment	*N*-Acetylneuraminic acid (μmoles/g. fresh wt. of tissue)	
	Particulate	Supernatant
None	1·54±0·28 (8)	0 (8)
Incubated (90 min., 37·5°)	0·79	0
Dialysed (4 hr., water)	0·37	0
Dialysed (16 hr., water)	0	0
Lysozyme (2 mg.; 30 min., 37·5°)	1·55	0
Lysolecithin (2 mg.; 30 min., 37·5°)	1·22	0
Lysolecithin (30 mg.; 30 min., 37·5°)	0·60	0
Trypsin (2 mg.; 3 hr., 37·5°)	1·20	0·06
Sodium deoxycholate (0·5%, pH 7·4)	0·60	0·03
EDTA (0·1 M, sodium salt)	1·19	0·34
Clupein sulphate (1 mg.)	0	0
Calf-thymus histone (1 mg.)	0	0

Table 8. *Affinity of subcellular fractions of guinea-pig cerebral cortex for basic proteins compared with their ganglioside content*

Subcellular fractions were prepared by Method 2 as outlined in the Methods section. The P1 fraction was washed three times and the P2A, P2B, P2C and P3 fractions were washed once with 0·14 M-NaCl and then resuspended in 0·14 M-NaCl and centrifuged at 105 400*g* for 30 min. Quantities (200 mg.) of each pellet were weighed into 2 ml. polythene Spinco tubes and 2 ml. of a solution of protamine (0·5 mg./ml.) or calf-thymus histone (1·0 mg./ml.) prepared in 0·01 M-phosphate buffer, pH 7·4, was added. The tubes were shaken for 5 min. to resuspend the particles and then centrifuged at 105 400*g* for 30 min. Portions (1 ml.) of the supernatants from the tubes were hydrolysed with 6 N-HCl and the arginine content was determined by the Sakaguchi reaction (see Wolfe & McIlwain, 1961). The results are expressed in μg. of arginine remaining in the supernatant and compared with protamine or histone solutions without addition of subcellular particles. Results for *N*-acetylneuraminic acid content of extracts of pellets are derived from Table 5.

Fraction	Moist wt. of pellet (mg./g. fresh wt. of tissue)	*N*-Acetylneuraminic acid			Arginine content (μg./ml.) of supernatant after addition of basic protein to pellet	
		μg./g. fresh wt. of tissue	μg./mg. of nitrogen	μg./200 mg. moist wt. of pellet	Protamine	Histone
P1	505	53	17	21	195	115
P2A	100	140	56	280	138	95
P2B	230	231	50	199	63	55
P2C	94	38	21	81	131	139
P3	100	112	86	224	30	30
Protamine alone	—	—	—	—	217	—
Histone alone	—	—	—	—	—	146

the total gangliosides extractable with no gain of gangliosides in the supernatant fraction (Table 7).

Ethylenediaminetetra-acetic acid. No effect of ethylenediaminetetra-acetic acid (EDTA) on the total amount of gangliosides was found. However, 22% of the total now occurred in the supernatant fraction. This is believed to be due to the greater difficulty in separating the supernatant fraction from the light, postmicrosomal membranes in the presence of EDTA. These postmicrosomal membranes have been shown to contain gangliosides (Table 1).

Basic proteins. When the protamine, clupein sulphate or calf-thymus histone was added to homogenates no gangliosides could be extracted subsequently from either the particulate or the supernatant fractions (Table 7).

Affinity of subcellular particles for basic proteins

The microsomal fraction has been shown to contain particles that form insoluble complexes with basic proteins (Wolfe & McIlwain, 1961). McIlwain (1960*a*, 1961) has produced evidence suggesting that extracted gangliosides form water-soluble, firmly bound complexes with basic proteins. In the tissue, however, gangliosides are present in water-insoluble cellular constituents and are rendered soluble only by certain extraction procedures. In Table 8 the removal of basic proteins from solution as insoluble complexes by

subcellular fractions is compared with the ganglioside content of these fractions. Fractions P2B and P3 show most affinity for basic proteins and also contain a high concentration of *N*-acetylneuraminic acid in the chloroform–methanol extracts. However, fraction P2A, although representing only 24% of the total tissue gangliosides, contains in a 200 mg. portion of the moist pellet a high concentration of gangliosides. This fraction does not show a corresponding affinity for basic proteins.

DISCUSSION

The experiments demonstrate that gangliosides are found mainly in the microsomal and postmicrosomal fractions of cerebral-cortex homogenates (Tables 1 and 3). However, when fractions were separated under conditions similar to those described by Hebb & Whittaker (1958), a large part of the gangliosides appeared in fraction P2 (Table 4). The centrifugal force used to separate this fraction (i.e. 22 500*g* for 20 min.) is considerably greater than is usually used for the separation of brain-mitochondrial fractions (see Brody & Bain, 1952; Hanzon & Toschi, 1959). Consequently fraction P2 contains particles which under conventional sedimentation procedures would occur in the microsomal fraction. When the particles of the P2 fraction were separated on the basis of density differences (Table 5) gangliosides were found mostly in particles of intermediate density (the P2B layer) and in least amount in the densest particles (the P2C layer). Studies of these fractions with the electron microscope (Whittaker, 1959; Gray & Whittaker, 1960) have shown that the P2A layer contains disrupted myelin fragments, the P2B layer contains pinched-off nerve endings, torn off during homogenizing, and the P2C layer contains mitochondria. The P2B fraction contains the synaptic vesicles enveloped within membranes, forming larger particles which appear identical with the terminal boutons seen in sections of cerebral cortex on the dendrites of neurones (Gray, 1959). It is therefore probable that gangliosides occur in fractions that contain fragments of cell membranes, particularly of dendrites. Parallel with this, the acidic mucopolysaccharides of bacteria containing muramic acid (3-*O*-α-carboxyethyl-D-glucosamine), a substance closely related to neuraminic acid, occur entirely within cell membranes (Strange & Kent, 1959; O'Brien, Glick & Zilliken, 1960).

It is now known that much of the microsomal fraction arises through fragmentation of the ergastoplasm during homogenizing and contains the endoplasmic reticulum and the ribonucleoprotein particles (Palay & Palade, 1955; Palade & Siekevitz, 1956; Porter, 1957). However, other cytological entities do occur. It is probable that the gangliosides occurring in the postmicrosomal particles are associated with the delicate fragments of membranes unattached to other subcellular constituents. It is unlikely that gangliosides are associated with the ribonucleoprotein particles in the microsomal fractions since EDTA in high concentrations has little effect on their distribution. Bivalent cations are required to maintain the integrity of these particles and chelating agents such as EDTA make them soluble (see Roberts, 1958).

The studies of McIlwain (1960*a*, *b*, 1961) have shown that ox-brain gangliosides combine with basic proteins. Harris & Saifer (1960*a*) subsequently noted that the metachromasia of strandin is suppressed by protamine sulphate, and these authors also have demonstrated a direct interaction between strandin and protamine by a turbidimetric procedure, finding it dependent on the ionic environment (Harris & Saifer, 1960*b*). The present investigation has also indicated an interaction between basic proteins and gangliosides. However, the presence of other acidic substances in subcellular fractions which may also combine with basic proteins must be considered. It should be noted that subfraction P2A, which apparently contains mainly myelin fragments, also contains gangliosides, and yet this fraction does not show a corresponding affinity for basic proteins (Table 8). At present this remains an anomaly. Klenk (1947) claimed that white matter contains negligible amounts of gangliosides, but Long & Staples (1959) reported a ganglioside content of white matter of about half that of grey matter for the rat brain. It is possible that the gangliosides associated with myelin elements may occur in less reactive combinations than do grey-matter gangliosides.

SUMMARY

1. Gangliosides occurred mainly in the microsomal fraction of homogenates of guinea-pig cerebral cortex separated by usual differential-centrifuging techniques. Within the microsomal fraction gangliosides were associated with two groups of particles differing considerably in sedimenting properties.

2. Ganglioside-rich particles were found in a subcellular fraction separating as a layer between 0·8 and 1·2 M-sucrose after equilibrium centrifuging in a density gradient.

3. Ox-brain gangliosides added to homogenates were recovered almost completely from the nuclear fraction.

4. Ultrasonic treatment of homogenates after removal of the nuclear fraction caused a shift in gangliosides to subcellular fractions containing

smaller particles but did not solubilize them. Much less gangliosides were extracted after ultrasonic treatment of homogenates containing nuclei.

5. The effect of added agents and various treatments to homogenates on the quantity of gangliosides extracted from particulate and supernatant fractions was studied.

6. Addition of protamine or histone completely prevented the extraction of gangliosides.

7. A comparison of the affinity of subcellular fractions for basic proteins with the content of gangliosides was made. The fractions containing most of the tissue gangliosides showed the greatest affinity for basic proteins except a fraction rich in myelin fragments.

8. The probable localization of gangliosides in cell membranes is discussed.

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Oestriol Metabolism by Rat- and Rabbit-Liver Slices

ISOLATION OF 2-METHOXYOESTRIOL AND 2-HYDROXYOESTRIOL

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When Marrian, Loke, Watson & Panattoni (1957) isolated 16α-hydroxyoestrone (3:16α-dihydroxyoestra-1:3:5-trien-17-one) from pregnancy urine, they suggested that this compound might be the intermediate in the conversion of oestrone (3-hydroxyoestra-1:3:5-trien-17-one) into oestriol (oestra-1:3:5-triene-3:16α:17β-triol). To test this suggestion, experiments were started to see if liver preparations could convert 16α-hydroxyoestrone into oestriol. Rat-liver slices could readily bring about this reduction but during the experiments the observation was made that the yield of oestriol was dependent on the gas phase above the incubation medium. When incubation was under air the yield of oestriol was 10% but increased to 22% on carrying out the incubation under nitrogen (R. J. B. King & G. F. Marrian, unpublished results). A number of other workers have found that oestrogen metabolism by rat-liver preparations is influenced by the gas phase used during the incubation (Ryan & Engel, 1953; Szego, 1953; Riegel & Mueller, 1954).

A number of new oestrogen derivatives have been isolated from biological material in recent years. These include 6′α′-hydroxyoestradiol-17β (oestra-1:3:5-triene-3:6′α′-17β-triol) and 6-oxo-oestradiol-17β (3:17β-dihydroxyoestra-1:3:5-trien-6-one)

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(Mueller & Rumney, 1957), 18-hydroxyoestrone (3:18-dihydroxyoestra-1:3:5-trien-17-one) (Loke, Marrian, Johnson, Mayer & Cameron, 1958) and 2-methoxy derivatives of oestrone (Kraychy & Gallagher, 1957; Engel, Baggett & Carter, 1957; Loke & Marrian, 1958), oestradiol-17β (oestra-1:3:5-triene-3:17β-diol) (Frandsen, 1959) and oestriol (Fishman & Gallagher, 1958). Furthermore, Levitz, Spitzer & Twombly (1958) have shown that [16-^{14}C]oestriol injected into women is in part excreted in the urine as 16-oxo-oestradiol-17β (3:17β-dihydroxyoestra-1:3:5-trien-16-one) and 16-*epi*-oestriol (oestra-1:3:5-triene-3:16β:17β-triol). In view of the number of possible metabolites, the oxidative metabolism of oestriol by liver preparations was further investigated. This paper describes the isolation and characterization of 2-hydroxyoestriol (oestra-1:3:5-triene-2:3:16α:17β-tetrol) and 2-methoxyoestriol (2-methoxyoestra-1:3:5-triene-3:16α:17β-triol) by incubating oestriol with rat- and rabbit-liver slices.

A preliminary account of the work published in this and the following paper has been presented to The Biochemical Society (King, 1960).

RESULTS

Oestriol metabolism of rat-liver slices and detection of two phenolic metabolites

The recovery of oestriol after incubation with rat-liver slices for 1 hr. in Krebs–Ringer phosphate was 55, 42 and 13% when incubated under nitrogen, air and oxygen respectively. The nitrogen used was unpurified commercial material and probably contained traces of oxygen. This would suggest that the amount of oestriol metabolized under completely anaerobic conditions may be even less than shown here.

One stage of the extraction procedure used in this preliminary experiment involved the extraction of the phenols into N-NaOH. As certain of the recently discovered oestrogen metabolites are alkali-labile (see Layne & Marrian, 1958), the alkali-treatment was omitted from the extraction procedure used in all the subsequent experiments.

When oestriol was incubated with liver slices under oxygen and the ether extract of this incubation was chromatographed on paper in the formamide–$CHCl_3$ system for 11 hr., two phenolic metabolites were detected. They were both non-ketonic. Metabolite I had a mobility intermediate between that of 16-*epi*oestriol and 17-*epi*oestriol (oestra-1:3:5-triene-3:16α:17α-triol), whereas metabolite II was more polar than oestriol and did not migrate from the origin even after development in this system for 48 hr. Metabolite II was anomalous in that it developed the Folin & Ciocalteu blue colour before the paper was suspended in the ammonia tank.

In the system acetic acid–water–ethylene dichloride (70:30:100, by vol.), I had the same mobility as 16-*epi*oestriol whereas II was more polar than oestriol.

Loke (1958) has detected a compound in the phenolic ketonic fraction of late-pregnancy urine which he suggested was 2-methoxy-16α-hydroxyoestrone (2-methoxy-3:16α-dihydroxyoestra-1:3:5-triene-17-one). On reduction of this compound with sodium borohydride, he obtained a product which, on his hypothesis, was 2-methoxyoestriol. This reduction product had the same chromatographic behaviour as I. This suggested that II might be 2-hydroxyoestriol.

Isolation of 2-methoxyoestriol after incubation of oestriol with rat-liver slices

Oestriol was incubated with rat-liver slices under oxygen in Krebs–Ringer phosphate solution, pH 7·4, for 4 hr., the medium was homogenized, extracted with ether and metabolite I separated from oestriol by paper chromatography in formamide–$CHCl_3$. After elution with methanol, I was considerably purified by discontinuous gradient elution from an alumina column with increasing amounts of methanol in benzene. The material eluted with 3% of methanol in benzene was crystallized from methanol–benzene and characterized as 2-methoxyoestriol by comparison of the melting point, optical rotation and infrared spectrum with the values quoted for 2-methoxyoestriol (Fishman & Gallagher, 1958).

The yield of crystalline 2-methoxyoestriol was 15·4 mg. from 550 mg. of oestriol. The yield of II was insufficient for complete characterization.

Isolation of 2-hydroxyoestriol after incubation of oestriol with rabbit-liver slices

Rabbit-liver slices could produce appreciable amounts of II but only trace amounts of I; ox-liver slices produced neither of these two metabolites. To facilitate the isolation of reasonable amounts of II, rabbit-liver slices were employed.

Metabolite II is unstable under oxidative conditions, especially in alkaline solution, and consequently anaerobic conditions were used wherever possible. II was isolated by ether extraction and chromatography on paper in acetic acid–water–ethylene dichloride (70:30:100, by vol.). After elution with methanol, II was exhaustively leached with ethyl acetate and the combined ethyl acetate-soluble material crystallized from aqueous 50% acetone. It was identified as 2-hydroxyoestriol by comparison of the melting point, ultraviolet and infrared spectra with those of authentic 2-hydroxyoestriol. From 190 mg. of oestriol, 1·3 mg. of crystalline 2-hydroxyoestriol was obtained.

Table 1. *Formation of 2-methoxyoestriol and 2-hydroxyoestriol by rat-liver slices*

Rat-liver slices (400 mg.), 4 ml. of Krebs–Ringer phosphate, pH 7·4, 200 μg. of oestriol, in 0·05 ml. of propylene glycol, were incubated under oxygen for 2 hr. at 37°. The figures are given as percentages of oestriol added.

	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
Slices boiled 1 min. before incubation	70	1	0	71
Viable slices (oestriol added just before extraction)	70	0	0	70
Viable slices	5	10	18	33
No tissue (oestriol added just before extraction)	72	0	0	72

Quantitative study of the conversion of oestriol into 2-hydroxyoestriol and 2-methoxyoestriol by rat-liver slices

Results of this experiment are shown in Table 1. Even though synthetic 6′α′-hydroxyoestriol (oestra-1:3:5-triene-3:6′α′:16α:17β-tetrol) and 6-oxo-oestriol (3:16α:17β-trihydroxyoestra-1:3:5-triene-6-one) (Marrian & Sneddon, 1960) were available, no trace of either of these compounds could be detected.

It can be seen from Table 1 that about 40% of the added oestriol has been converted into undetected metabolites. Crepy (1947) has shown that guinea-pig- and rabbit-liver slices are capable of forming oestriol glucuronide. As this might account for some of the lost oestriol, the aqueous phase, after ether extraction of the free oestrogens, was extracted twice with 50 ml. portions of butan-1-ol and the butanol was evaporated to dryness under vacuum. The residue was hydrolysed with β-glucuronidase for 48 hr. and then extracted with ether and the free steroids were estimated as before. No oestriol, 2-methoxyoestriol or 2-hydroxyoestriol was liberated by this procedure.

EXPERIMENTAL

Preparation of liver slices and incubation medium. Rats and rabbits were killed by a blow on the back of the neck. The livers were rapidly removed and kept on ice until used. In the preparative-scale experiments the tissue slices were prepared with a Stadie–Riggs hand microtome, but in the other experiments the slices were cut free-hand to a thickness of about 0·5 mm. The slices were washed in ice-cold Krebs–Ringer phosphate, blotted dry and weighed.

Krebs–Ringer phosphate, pH 7·4, was prepared by the method of Krebs & Henseleit (1932). It was made up fresh from its component solutions for each experiment. The required gas was bubbled through this solution for at least 30 min. before the experiment was started. In the quantitative studies the gas was played on the surface of the incubation medium for 30 sec. and the tubes were immediately stoppered.

Paper chromatography. All papers were washed for 72 hr. in a Soxhlet apparatus with methanol–$CHCl_3$ (1:1, v/v) and then dried at 37°. Chromatography was carried out in a thermostatically controlled room at 20±2°.

Table 2. *Chromatographic behaviour of a number of oestriol derivatives in acetic acid–water–ethylene dichloride* (70:30:100, *by vol.*)

Compound	R_F
16-*epi*Oestriol	0·7
Oestriol	0·6
6-Oxo-oestriol	0·4
2-Hydroxyoestriol	0·2
6′α′-Hydroxyoestriol	0·1

Analytical and preparative-scale chromatograms with formamide–$CHCl_3$ were carried out as described by Layne & Marrian (1958).

For the quantitative separation and isolation of 2-hydroxyoestriol the material was dissolved in methanol and transferred to Whatman 3 MM paper. After equilibration overnight in the chromatography tank, the paper was developed for about 3 hr. with acetic acid–water–ethylene dichloride (70:30:100, by vol.). About 10 μg. of standard 2-hydroxyoestriol was run on each paper. This strip was cut out, sprayed with diluted Folin & Ciocalteu reagent and suspended in a tank containing NH_3 solution to locate the steroid. The corresponding areas of the main part of each paper were cut out and eluted with methanol in the apparatus designed by Saffran & Jarman (1960). The methanol was evaporated under nitrogen. The remainder of the paper was sprayed with Folin & Ciocalteu reagent to confirm that no oestriol was contaminating the 2-hydroxyoestriol area. When this system was used on a preparative scale, the material was applied to a large number of 14 cm. × 35 cm. strips of 3 MM paper as a band along the origin.

The average R_F of other oestrogen derivatives in this system with Whatman 3 MM paper are given in Table 2 (Whatman no. 1 paper may also be used). To obtain reproducible results with this solvent system rigid standardization of the procedure is essential.

Other chromatographic methods. Celite columns (1 cm. × 10 cm.), with the system methanol–water–ethylene dichloride (70:30:100, by vol.), were prepared by the method of Bauld (1955). In this system 2-methoxyoestriol is eluted with 4–11 ml. and oestriol with 11–35 ml. of ethylene dichloride. Alumina (Savory and Moore Ltd.) was used.

Melting points. All melting points were determined in sealed evacuated tubes and are uncorrected for emergent stem.

Girard reaction. The dry residue was dissolved in 1 ml. of ethanol and 1 ml. of acetic acid. Girard T reagent (5–10 mg.) (Girard & Sandulesco, 1936) was added and the solution allowed to stand overnight at room temperature. The vessel was chilled and 90% neutralized with cold N-NaOH. About 2 g. of NaCl was added and the volume made up to 25 ml. with cold water. This was extracted three times with 25 ml. of ether and the combined ether extract was washed once with 5 ml. of water. The ether was further washed twice with 5 ml. of 5% (w/v) $NaHCO_3$ and once with 5 ml. of water. The ether (non-ketonic) fraction was evaporated to dryness under vacuum.

The aqueous phase was acidified with 6 ml. of conc. HCl and left at room temperature for 1 hr. to hydrolyse the Girard complex. The solution was then extracted with ether and washed with $NaHCO_3$ and water as before to give the ketonic fraction.

Steroids. The oestriol, 16-*epi*oestriol, 6-oxo-oestriol and 6′α′-hydroxyoestriol were generous gifts from Dr G. F. Marrian, F.R.S. The 2-hydroxyoestriol was synthesized by Dr M. M. Coombes by a modification of the method used by Fishman (1958) to prepare 2-methoxyoestrogen derivatives. 2-Hydroxyoestradiol-17β (oestra-1:3:5-triene-2:3:17β-triol) was donated by Dr G. C. Mueller. The oestriol was added to the incubation flasks as a solution in propylene glycol.

Solvents. Ether (A.R.) was distilled once just before use. Other solvents and Celite were purified by the methods described by Bauld (1953).

β-Glucuronidase hydrolysis. To each flask were added 5 ml. of M-acetate buffer, pH 4·6, and 5×10^3 units (Fishman, Springer & Brunetti, 1948) of soluble β-glucuronidase obtained from *Patella vulgata* (Dodgson & Spencer, 1953). The flasks were stoppered and incubated at 37° for 2 days.

Estimation of steroids. Oestriol was estimated by means of the Kober reaction as modified by Brown (1952) and Bauld (1954). A modification of the method of Davies & Mitchell (1954) was used to estimate 2-hydroxyoestriol. M-Sodium carbonate (1·5 ml.) and 1·5 ml. of Folin & Ciocalteu reagent (diluted 1:4 with water) were added to the dry residue and incubated at 37° for 20 min. The resulting blue colour was read in a Unicam SP. 600 spectrophotometer at 745 mμ.

When 2-methoxyoestriol is heated in boiling water for 4 min. with Bauld's (1954) 'oestriol reagent' and then cooled in cold water, the resulting red colour can be used to estimate the 2-methoxyoestriol. The colour has an absorption maximum at 522 mμ, is stable for at least 20 min. at room temperature and obeys Beer's Law up to a concentration of 40 μg. of steroid/3 ml. of reagent.

Extraction of steroids. In the preliminary experiments the following extraction procedure was used. The incubation medium plus liver slices were homogenized, 5 ml. of 2N-HCl was added, and the mixture extracted three times with 50 ml. of ether. The combined ether extract was washed twice with 20 ml. of 5% (w/v) $NaHCO_3$ and then extracted three times with 20 ml. of N-NaOH. The alkali was partly neutralized with 10 ml. of 5N-H_2SO_4 and then brought to pH 8–10 by bubbling CO_2 through the solution. The pH was checked with phenolphthalein indicator paper. Sodium chloride (10 g.) was added to prevent emulsification and the mixture extracted three times with 50 ml. of ether. The ether extract was washed twice with 20 ml. of water and then evaporated to dryness under vacuum.

In all of the other experiments the $NaHCO_3$ wash and phenol separation were omitted so that the initial ether extract was evaporated to dryness directly.

Separation of 2-methoxyoestriol, oestriol and 2-hydroxyoestriol

The dry ether-soluble residue was dissolved in 2·5 ml. of methanol and a 1 ml. fraction of this used to estimate 2-hydroxyoestriol as described above. Another 1 ml. portion was evaporated to dryness on a warm-water bath with the aid of an air blast. The contaminating pigment was removed from this residue by discontinuous gradient elution from an alumina column (0·7 cm. × 6 cm.) deactivated with 2% water. The material eluted with 20 ml. of 1% methanol in benzene was discarded. The solvent was then changed to 4% methanol in benzene and 50 ml. of this solvent collected as the combined 2-methoxyoestriol and oestriol fraction. This was evaporated to dryness on a warm-water bath and the residue was dissolved in 2·5 ml. of ethylene dichloride. A portion (2 ml.) of this was transferred to a Celite column to separate the 2-methoxyoestriol and oestriol as described above. All of the 2-hydroxyoestriol is destroyed by this procedure.

Regular 70–75% recoveries of added oestriol could be obtained by this method, and the recovery of 2-methoxyoestriol was almost quantitative.

Large-scale isolation of metabolites I and II and their identification as 2-methoxyoestriol and 2-hydroxyoestriol

Oestriol (150 mg.) dissolved in 35 ml. of propylene glycol was incubated with 125 g. of rat-liver slices in 1·2 l. of Krebs–Ringer phosphate, pH 7·4, for 4 hr. The incubations were carried out under oxygen in a series of 250 ml. stoppered conical flasks. The flasks were reoxygenated after 2 hr. At the end of the incubation the contents of all the flasks were combined and homogenized in a Waring Blendor. Hydrochloric acid (250 ml.; 2N) was added and the mixture extracted four times with 1 l. of ether. The ether extract was evaporated to dryness on a boiling-water bath. The resulting brown oil was extracted four times with 30 ml. of aqueous 70% methanol and the methanol was washed twice with 30 ml. of *n*-hexane. The aqueous methanol was evaporated to dryness under vacuum. The resulting brown oil was transferred to a large number of Whatman no. 42 papers and chromatographed in the formamide–$CHCl_3$ system (about 15 mg. of oil/15 cm.-wide paper) for 19 hr. The mobile phase which had run off the bottom of the papers was collected and evaporated to dryness under vacuum. This was called the 'eluate fraction'.

A complete separation of I from oestriol was achieved by this method, but the separation of oestriol and metabolite II was not satisfactory. The fraction containing II was rechromatographed in the same system for 60 hr. to remove the oestriol. Metabolites I and II were eluted with methanol to yield 25 mg. of I and 6 mg. of II. Another 400 mg. of oestriol was processed in the same way to give a further 71 mg. of I and 27 mg. of II.

Metabolite I was contaminated with a greenish-brown pigment which was removed by alumina chromatography.

The alumina was deactivated with 2% of water and a 1·5 cm. × 5 cm. column was prepared in benzene. Half of I was transferred to this column with benzene and eluted with increasing amounts of methanol in benzene. Portions (50 ml. each) of 0·5, 1·0 and 1·5% of methanol in benzene did not elute any of metabolite I but it was all eluted with 250 ml. of 3% of methanol in benzene. The other half of I was treated in the same way and the two parts were combined to yield 54 mg. of a yellowish oil. It was leached twice with 0·1 ml. of acetone to remove some of the yellow pigment. Two crystallizations from methanol–benzene did not completely remove an oily impurity but a further leaching with two portions of 0·1 ml. of acetone and subsequent crystallization from methanol–benzene gave 15·4 mg. of white crystals. These had m.p. 213–215°; $[\alpha]_D^{17}$ +81·1° in ethanol. The values quoted by Fishman & Gallagher (1958) for 2-methoxyoestriol are: m.p. 215–218°; $[\alpha]_D^{18}$ +83° in ethanol. The infrared spectrum of I was identical with that of 2-methoxyoestriol. The u.v. spectrum of I (ethanol $\lambda_{max.}$ 287 mμ, ε 3980; $\lambda_{min.}$ 253 mμ, ε 676) differed slightly from that of 2-methoxyoestriol (ethanol $\lambda_{max.}$ 286 mμ, ε 3700; $\lambda_{min.}$ 253 mμ, ε 350). This could be due to a trace impurity.

The batch of II obtained with rat-liver slices was impure and did not contain sufficient material to allow a complete characterization. This material was partially purified by precipitation from aqueous ethanol and was used for the preliminary experiments on the structure of this metabolite. The u.v.-absorption curve in ethanol showed a maximum at 287–289 mμ and a minimum at 254 mμ, which suggested that it was related to 2-methoxyoestriol. The colour reactions with Folin & Ciocalteu reagent, ammoniacal silver nitrate and 10% (w/v) ethylenediamine in aqueous ammonia (ammonia–water, 2:8) (Weil-Malherbe & Bone, 1957) were all compatible with II being a catechol. It was unstable under oxidative conditions, especially in alkali.

To facilitate the isolation of II, female rabbit-liver slices were used to prepare the next batch of II. The steroid: tissue:salt solution ratios were the same as had been used in the experiments with rats.

The incubation medium was extracted as before but the separation of oestriol and II was achieved by chromatography on a number of sheets of Whatman 3 MM paper in acetic acid–water–ethylene dichloride (70:30:100, by vol.) for 4 hr. The fraction containing II was eluted with methanol and rechromatographed in the same system for 5 hr. From 192 mg. of oestriol, 120 mg. of crude II was obtained as a dark-brown solid. This was exhaustively leached with 1 ml. portions of ethyl acetate and the ethyl acetate-soluble material was taken to dryness under nitrogen. The pale-yellow solid was washed three times with 0·5 ml. of ethyl acetate to remove most of the contaminating pigment. This left 19 mg. of white solid. This was crystallized twice from aqueous 50% methanol at 0°, the crystals being washed three times with 0·1 ml. of the same solvent after each crystallization. This gave 1·3 mg. of white crystals, which had m.p. 267–269° with shrinkage at 263°. The mixed m.p. with 2-hydroxyoestriol (m.p. 269–271°) was 265–268°.

The infrared spectrum of this material was virtually identical with that of authentic 2-hydroxyoestriol and the difference could be explained by the presence of a small amount of ketonic material absorbing at 1750 $cm.^{-1}$

Metabolite II obtained with rabbit-liver slices had the same chromatographic behaviour, colour reactions, instability to alkali and u.v.-absorption curve as the material obtained with rat liver.

Attempted detection of other phenolic oestriol metabolites

The 'eluate fraction' obtained from 100 mg. of oestriol was chromatographed in formamide–$CHCl_3$ for 6 hr. No 16α-hydroxyoestrone or 16-oxo-oestradiol-17β could be detected.

Any 16-*epi*oestriol which might have been formed from oestriol would have been isolated with the 2-methoxyoestriol. The mother liquors from the isolation of 2-methoxyoestriol were combined to give 40 mg. of brown oil. Although 16-*epi*oestriol and 2-methoxyoestriol cannot be separated adequately by chromatographic methods, acetonide formation would distinguish these two compounds. The method used was that of H. J. Blair & J. B. Brown (personal communication). The brown oil was dissolved in 1 ml. of methanol plus 1 ml. of acetone. Two drops of conc. HCl were added and the solution was left at room temperature for 1 hr. The solution was evaporated to dryness under vacuum and the residue dissolved in 20 ml. of 0·1 N-NaOH. This was extracted three times with 20 ml. portions of $CHCl_3$ and the $CHCl_3$ washed twice with 10 ml. of water, the water washings being added to the alkaline phase. The $CHCl_3$ was dried with Na_2SO_4 and evaporated to dryness under vacuum to yield 5·7 mg. of 'acetonide fraction'.

Carbon dioxide was bubbled through the alkali until neutral to phenolphthalein and then extracted three times with 20 ml. portions of ether. The ether was washed twice with 20 ml. of water and evaporated to dryness to give 23·3 mg. of 2-methoxyoestriol fraction.

A portion (500 μg.) of the 'acetonide fraction' was hydrolysed with 10 ml. of 5% (v/v) HCl on a boiling-water bath for 30 min. The hydrolysate was extracted three times with 30 ml. portions of ether and the ether washed once with 20 ml. of water. The ether was evaporated to dryness to give the 'hydrolysed acetonide fraction'. Portions (30 μg.) of the hydrolysed and unhydrolysed acetonide fractions were chromatographed in formamide–$CHCl_3$ for 11 hr. Both fractions ran as single compounds with the same mobility as 2-methoxyoestriol even though there was a separation of standard 2-methoxyoestriol from standard 16-*epi*-oestriol.

The appearance of 2-methoxyoestriol in the 'acetonide fraction' is probably explained by the observation of Engel *et al.* (1957) that the 2-methoxy derivative of oestrone is not completely extracted from toluene by N-NaOH.

Although the chromatographic systems used could separate oestriol, 6-oxo-oestriol, 2-hydroxyoestriol and 6′α′-hydroxyoestriol from each other, no 6-oxo-oestriol or 6′α′-hydroxyoestriol could be detected.

DISCUSSION

This demonstration of a 2-methoxylating system in rat and rabbit liver implicates this organ as the site of formation of the urinary 2-methoxyoestrogen derivatives. Such derivatives of oestrone (Kraychy & Gallagher, 1957; Engel *et al.* 1957;

Loke & Marrian, 1958), oestradiol-17β (Frandsen, 1959), oestradiol-17α (oestra-1:3:5-triene-3:17α-diol) (Stimmel, 1959), oestriol (Fishman & Gallagher, 1958) and possibly 16α-hydroxyoestrone and 16-oxo-oestradiol-17β (Loke, 1958) have been isolated from human urine under various conditions, which would indicate that 2-methoxylation is a fairly general metabolic reaction.

The isolation of 2-hydroxy- and 2-methoxyoestriol suggests that methoxylation is a two-stage process, the initial 2-hydroxylation being followed by methylation of the 2-hydroxyl group. This is supported by the experiments with cell-free preparations described in the following paper and by the conversion of 2-hydroxyoestradiol-17β into 2-methoxyoestrone (2-methoxy-3-hydroxyoestra-1:3:5-trien-17-one) *in vivo* (Axelrod, Rao & Goldzieher, 1960).

To date no 2-hydroxylated oestrogens have been isolated from urine, but Garst & Friedgood (1952) have put forward some slender evidence that such compounds might in fact be present in urine and it is known that after the injection of [16-^{14}C]oestradiol-17β (Beer & Gallagher, 1955) or [16-^{14}C]-oestrone (Midgeon, Wall & Bertrand, 1959) into humans, an appreciable amount of the urinary radioactivity is associated with a fraction more polar than oestriol.

It can be seen from the figures quoted in Table 1 that an appreciable amount of the added oestriol has been 'lost'. This might be due to the formation of ether-soluble, non-phenolic compounds such as 2:3-dimethoxyoestra-1:3:5-triene:16α:17β-diol or ring A saturated derivatives.

It seems more probable, however, that the oestriol was metabolized to ether-insoluble compounds. A number of workers (Szego, 1953; Riegel & Mueller, 1954; Sandberg, Slaunwhite & Antoniades, 1957) have shown that oestrone and oestradiol-17β can be irreversibly bound to liver proteins, and it would seem likely that a similar reaction is occurring with oestriol in the experiments described in this paper. Bhargava, Hadler & Heidelberger (1955) have demonstrated that the protein-binding of 1:2:5:6-dibenzanthracene results in the rupture of one of the anthracene rings to produce a muconic acid derivative. It may be that oestrogens can be bound via ring A in a similar manner.

Crepy (1947) has shown that guinea-pig and rabbit-liver slices can form oestriol glucuronide from oestriol, but no such conjugates could be detected in the experiments described in this paper. This is in agreement with the findings of Lieberman, Tagnon & Schulman (1952).

SUMMARY

1. The metabolism of oestriol by rat-liver slices has been shown to be stimulated by oxygen and inhibited by nitrogen. These results have led to the detection of two previously unknown metabolites of oestriol *in vitro*. These have been characterized as 2-methoxyoestriol and 2-hydroxyoestriol by melting points and infrared spectrometry. The 2-methoxyoestriol has been further characterized by optical rotation and ultraviolet spectroscopy.

2. No other phenolic metabolites of oestriol could be detected.

3. With rat-liver slices, the yields of 2-methoxyoestriol and 2-hydroxyoestriol are 10 and 18% respectively. Rabbit-liver slices produce more of the 2-hydroxy compounds but less 2-methoxyoestriol than does rat liver, whereas ox liver produces neither of these metabolites.

4. With rat-liver slices, about 40% of the oestriol is unaccounted for. No glucuronides could be detected. The possible fate of this oestriol is discussed.

The author is greatly indebted to Dr G. F. Marrian, F.R.S., for his guidance and help in this work; to Dr M. M. Coombes (Imperial Cancer Research Fund Laboratories) for undertaking the synthesis of 2-hydroxyoestriol and for determining the infrared spectrum of this compound; to Dr T. F. Gallagher (Sloan-Kettering Institute for Cancer Research) for determining the infrared spectrum of 2-methoxyoestriol; and to Dr J. W. Minnis for his microanalytical work.

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Metabolism of Oestriol *in vitro*

COFACTOR REQUIREMENTS FOR THE FORMATION OF 2-HYDROXYOESTRIOL AND 2-METHOXYOESTRIOL

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The conversion of oestriol into 2-hydroxyoestriol and 2-methoxyoestriol by rat- and rabbit-liver slices described in the preceding paper indicated a new metabolic pathway for oestrogens *in vitro*. The results suggested that the oestriol was hydroxylated in the 2 position and this hydroxyl group was then methylated to form 2-methoxyoestriol.

A number of papers (reviewed by Grant, 1956*a*) have described the hydroxylation of neutral steroids *in vitro*, and Mueller & Rumney (1957) have studied the 6′α′-hydroxylation of oestradiol-17β by rat- and mouse-liver microsomal preparations. These hydroxylations all appear to require molecular oxygen and reduced pyridine nucleotide. The 2-hydroxylation of oestriol represents a major difference from these reactions in that the hydroxyl group is entering a benzenoid ring structure. Brodie *et al.* (1955) have shown that the hydroxylation of aromatic compounds such as acetanilide also requires oxygen and reduced triphosphopyridine nucleotide, so that some similarity exists between the hydroxylation of aromatic and aliphatic compounds. However, Kaufman (1959) has shown that a tetrahydrofolic acid derivative is required for the conversion of phenylalanine into tyrosine, whereas this vitamin has no effect on the 11β-hydroxylation of 11-deoxycorticosterone (Tomkins, Curran & Michael, 1958).

The *O*-methylation of catechols is well known (Axelrod & Tomchick, 1958; Pellerin & D'Ionio, 1958), and, like *N*-methylation (Cantoni, 1951; Cantoni & Vignos, 1954), the methyl group can be derived from methionine via *S*-adenosylmethionine.

This paper describes the cofactor requirements of the rat-liver 2-methoxylating system.

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EXPERIMENTAL

Enzyme preparations and methods

The rats were killed by a blow on the back of the neck. Liver and kidney homogenates were prepared by mincing the tissue in a Latapie mincer to remove connective tissue and then homogenizing with cold 0·25M-sucrose in a glass homogenizer fitted with a nylon pestle. The ovaries were disrupted in a 0·8 cm. × 15 cm. glass tube with a hand-operated metal plunger; usually, 20% (w/v) homogenates were used. The uteri were slit longitudinally and the inside surface was blotted dry with filter paper. They were then cut into small pieces and finely minced with a Mickle automatic slicer. This brei was added to the appropriate volume of 0·25M-sucrose to give a 20% (w/v) suspension.

When required, the cell debris and nuclei were removed by centrifuging for 10 min. at 700*g* at 0° in a MSE refrigerated centrifuge and the mitochondria by centrifuging

for 10 min. at 7000*g*. The 7000*g* supernatant fraction was usually obtained by centrifuging the whole homogenate for 10 min. at 7000*g*. The 105000*g* supernatant was separated from the microsomes by centrifuging the 7000*g* fraction for 30 min. at 105000*g* in a preparative Spinco model L centrifuge. The microsomes were suspended in 0·25 M-sucrose by gentle homogenization with a glass ball so that 1 ml. of the suspension was equivalent to 1 ml. of homogenate (equivalent to 200 mg. wet wt. of original tissue).

Duplicate incubations were carried out in glass-stoppered test tubes and each experiment was repeated at least once.

The extraction and estimation of steroids were carried out as described in the preceding paper (King, 1961) except that the alumina-chromatography step was omitted in the experiments in which 2-methoxyoestriol was not estimated. By this modified method 80% recoveries of oestriol could be obtained regularly.

The Fast Black Salt K (diazotized *p*-nitrophenylazodimethoxyaniline) derivatives were prepared by the method of Heftmann (1950). The Girard reaction was carried out as described in the preceding paper. The sodium borohydride reduction was carried out by dissolving the dry material in 1 ml. of methanol and adding 2 mg. of sodium borohydride (British Drug Houses Ltd.). This solution stood at room temperature for 1 hr. and was then diluted with 10 ml. of 2 N-HCl plus 39 ml. of water. The resultant solution was extracted three times with portions (50 ml.) of ether and the ether washed twice with 20 ml. of water. The ether extract was evaporated to dryness under vacuum.

Materials

Diphosphopyridine nucleotide (DPN) and barium glucose 6-phosphate were obtained from C. F. Boehringer und Söhne (GmbH., Mannheim, Germany). Sodium glucose 6-phosphate solutions were prepared from the barium salt by precipitating the barium with the calculated amount of sodium sulphate. Calcium lactate was kindly donated by Dr J. K. Grant and the sodium salt prepared from this by addition of the calculated amount of sodium oxalate. Triphosphopyridine nucleotide (TPN), glucose 6-phosphate dehydrogenase and the sodium salt of adenosine triphosphate (ATP) were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). L-Methionine and folic acid were bought from British Drug Houses Ltd. Dihydrofolic acid was kindly prepared by Dr E. Brode by the method of Futterman (1957). Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid. Adams platinum oxide catalyst (Johnson, Matthey and Co.) (30 mg.) was suspended in 10 ml. of acetic acid and hydrogenated for 3 hr. at room temperature with gentle shaking. Folic acid (100 mg.) was then added and the shaking continued until the yellow suspension changed to a green solution. The catalyst was removed by centrifuging and the tetrahydrofolic acid precipitated with 100 ml. of dry ether. The tetrahydrofolic acid was centrifuged off and rapidly washed twice with portions (50 ml.) of dry ether. The whitish precipitate was dried *in vacuo* and stored under nitrogen in the dark at −17°.

In addition to the steroids mentioned in the preceding paper, the stilboestrol (3:4-di-*p*-hydroxyphenylhex-3-ene) and 17α-ethynyloestradiol-17β (17α-ethynyloestra-1:3:5-triene-3:17β-diol) were gifts from Dr G. S. Boyd. The oestriol was added to the incubation tubes as a solution in propylene glycol.

Ether (A.R. grade) was distilled once immediately before use. Other solvents and Celite were purified by the methods described by Bauld (1953).

RESULTS

Cofactor requirements for the formation of 2-methoxyoestriol by liver homogenates

Magnesium ions, ATP, L-methionine, DPN and TPN were required for the optimum conversion of oestriol into 2-methoxyoestriol. The pyridine nucleotide requirements of this reaction are shown in Table 1; in the absence of DPN and TPN, the amount of oestriol metabolized is small and very little, if any, 2-hydroxyoestriol is produced. Although the addition of oxidizable substrates (lactate and glucose 6-phosphate) has very little effect, this is probably due to the presence of endogenous substrates which can reduce DPN and TPN, as the experiment described in Table 7 indicates that the reduced form is required for the 2-hydroxylation reaction.

Table 1. *Effect of di- and tri-phosphopyridine nucleotide on metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0·25 M-sucrose, 150 μmoles of $MgCl_2$, 5 μmoles of ATP, 60 μmoles of L-methionine, 192 μmoles of potassium phosphate, pH 7·4, 240 μmoles of KCl and 0·52 μmole (150 μg.) of oestriol in 0·035 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37°. G-6-P, Glucose 6-phosphate.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
None	63	0	1	64
0·7 μmole of DPN + 4·5 μmoles of lactate	51	4	1	56
1·4 μmoles of DPN + 9 μmoles of lactate	42	5	1	48
0·7 μmole of TPN + 4·5 μmoles of G-6-P	37	9	5	51
1·4 μmoles of TPN + 9 μmoles of G-6-P	7	11	6	24
0·7 μmole of DPN + 0·7 μmole of TPN + 4·5 μmoles of lactate + 4·5 μmoles of G-6-P	17	18	6	41
0·7 μmole of DPN + 0·7 μmole of TPN	19	15	5	39
0·7 μmole of DPN + 0·7 μmole of TPN (oestriol added just before extraction)	73	0	0	73

The effect of adding Mg^{2+} ions, ATP and L-methionine is shown in Table 2. No other methyl donors were tested. These three cofactors have a marked effect on the production of 2-methoxyoestriol without altering the 2-hydroxylase activity. There is an absolute requirement for Mg^{2+} ions and L-methionine, whereas ATP has a pronounced stimulating effect. Dialysis of the homogenate against water at 0° for 18 hr. resulted in the complete loss of 2-hydroxylase activity, whereas preincubation of the homogenate for 30 min. at 37° resulted in the complete loss of methylating activity and a partial loss of the hydroxylase system (Table 3).

Conversion of 2-hydroxyoestriol into 2-methoxyoestriol

When 2-hydroxyoestriol was incubated with the optimum methoxylating system described in Table 2 there was a 20–30% conversion into 2-methoxyoestriol. The omission of DPN and TPN from the incubation medium had no effect, but in the absence of L-methionine no 2-methoxyoestriol was formed.

Substrate specificity of the 2-methoxylating system

Two incubation tubes were set up for each compound to be tested. The complete methoxylating system described in Table 2 was added to tube A of each pair and L-methionine and ATP were omitted from the B series of tubes. The required compound (150 μg.) was added to the incubation tubes as a methanolic solution (0·025 ml.). Oestrone, oestradiol-17β, 17α-ethynyloestradiol-17β and stilboestrol were used as substrates. After incubation for 1 hr. at 37° an ether extract was prepared as before and divided into three equal parts.

Part 1 was chromatographed on Whatman no. 1 paper in methanol–water–benzene–hexane

Table 2. *Effect of magnesium ions, adenosine triphosphate and L-methionine on metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0·25 M-sucrose, 0·7 μmole of DPN, 4·5 μmoles of sodium lactate, 0·7 μmole of TPN, 4·5 μmoles of glucose 6-phosphate, 192 μmoles of potassium phosphate, pH 7·4, 240 μmoles of KCl and 0·52 μmole (150 μg.) of oestriol in 0·035 ml. of propylene glycol. Where stated, the following additions were made: 5 μmoles of ATP, 150 μmoles of $MgCl_2$, 150 μmoles of $CaCl_2$, 60 μmoles of L-methionine. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37°.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
150 μmoles of $MgCl_2$	20	0	16	36
150 μmoles of $MgCl_2$ + 60 μmoles of L-methionine	25	6	8	39
150 μmoles of $MgCl_2$ + 5 μmoles of ATP	23	0	16	39
150 μmoles of $CaCl_2$ + 5 μmoles of ATP + 60 μmoles of L-methionine	30	0	15	45
150 μmoles of $MgCl_2$ + 5 μmoles of ATP + 60 μmoles of L-methionine	19	15	5	39
150 μmoles of $MgCl_2$ + 5 μmoles of ATP + 60 μmoles of L-methionine (oestriol added just before extraction)	73	0	0	73

Table 3. *Effect of adenosine triphosphate on the metabolism of oestriol by rat-liver homogenates*

Figures are given as percentages of oestriol added. All incubations contained 0·7 μmole of DPN, 0·7 μmole of TPN, 4·5 μmoles of sodium lactate, 4·5 μmoles of glucose 6-phosphate, 60 μmoles of L-methionine, 150 μmoles of $MgCl_2$, 240 μmoles of KCl and 192 μmoles of potassium phosphate, pH 7·4, and 0·52 μmole (150 μg.) of oestriol in 0·035 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°. Where stated, the homogenate in sucrose was preincubated for 30 min. at 37° without any additions. 20% (w/v) Homogenates in 0·25 M-sucrose were used.

Additions	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
1 ml. of homogenate (oestriol added just before extraction)	72	0	0	72
1 ml. of homogenate	14	3	14	31
1 ml. of homogenate + 5 μmoles of ATP	10	15	8	33
1 ml. of preincubated homogenate	41	0	10	51
1 ml. of preincubated homogenate + 5 μmoles of ATP	19	0	11	30

(70:30:50:50, by vol.) for 3½ hr. With the compounds used in this experiment, this system would separate any *o*-methoxy derivative from the parent phenol. In all of the A series of tubes there was a Folin & Ciocalteu-positive compound which was less polar than the original phenol. With oestrone, this compound had the same mobility as 2-methoxyoestrone. In the A tube of the oestradiol-17β incubation two compounds were detected which had the same mobility as 2-methoxyoestrone and oestrone respectively. In all of the B series of tubes these less-polar metabolites were absent, apart from the conversion of oestradiol-17β into oestrone.

Part 2 was chromatographed on Whatman no. 42 paper in the formamide–chloroform system for 10 hr. No compound more polar than oestradiol-17β was detected in either of the oestrone incubations. In the B but not in the A oestradiol-17β incubation there was a compound which turned blue with Folin & Ciocalteu reagent in the absence of alkali and which had the same mobility as 2-hydroxyoestradiol-17β. In the B tube of the 17α-ethynyloestradiol-17β incubation, and in both stilboestrol incubations, there were metabolites slightly more polar than 16-*epi*oestriol which, from their chromatographic behaviour and reaction with Folin & Ciocalteu reagent, could have been the *O*-hydroxylated derivatives. With stilboestrol there was about twice as much of this metabolite as in the A tube.

Part 3 was chromatographed on Whatman no. 1 paper for 4½ hr. in acetic acid–water–ethylene dichloride (70:30:100, by vol.) but no phenols more polar than oestriol could be detected.

Metabolism of oestriol by homogenates of different rat tissues

Table 4 shows that the 2-hydroxylase is absent in rat kidney, uterus and ovary and that there is no sex difference in the liver 2-hydroxylase activity. The large amount of oestriol metabolized by kidney homogenates is partly explained by the formation of 16-oxo-oestradiol-17β and 16-*epi*oestriol (King, 1960).

In this and subsequent experiments a simple potassium phosphate buffer was used instead of the potassium phosphate–magnesium chloride–potassium chloride solution employed in the methoxylation studies. When the phosphate buffer was replaced by an equal volume of 0·35 M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)–HCl, pH 7·4, no 2-hydroxyoestriol was formed. This hydroxylase system thus differs from the 11β-hydroxylase from ox-adrenal mitochondria which is more active in tris than in phosphate (Grant, 1956*b*).

Intracellular localization of the liver 2-hydroxylase system

The results of experiments with different cell fractions are shown in Table 5. Removal of the

Table 4. *Metabolism of oestriol by rat-tissue homogenates*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 20% (w/v) homogenate in 0·25 M-sucrose, 0·7 μmole of DPN, 0·7 μmole of TPN, 4·5 μmoles of sodium lactate, 4·5 μmoles of glucose 6-phosphate, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Tissue	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
Female liver (oestriol added just before extraction)	81	0	81
Female liver	16	27	43
Male liver	14	24	38
Female kidney	37	1	38
Uterus	76	1	77
Ovary	70	1	71

Table 5. *Intracellular distribution of rat-liver 2-hydroxylase*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of the cell fraction in 0·25 M-sucrose, 0·7 μmole of DPN, 0·7 μmole of TPN, 4·5 μmoles of sodium lactate, 4·5 μmoles of glucose 6-phosphate, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Cell fraction	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
Whole homogenate	16	27	43
Minus 700*g* sediment	9	30	39
Minus 7000*g* sediment	32	16	48
Minus 7000*g* sediment + 3 μmoles of ATP	4	29	33

nuclei and cell debris by a short centrifuging at 7000*g* has no effect on the pattern of oestriol metabolism, but subsequent removal of the mitochondria appreciably lowers the 2-hydroxylase activity. The activity of this 7000*g* supernatant fraction could be restored to that of the whole homogenate by the addition of ATP. The results shown in Tables 2 and 3 indicate that ATP does not affect the 2-hydroxylase activity of whole homogenates. The presence of ATP or an ATP-synthesizing system in the homogenate is suggested by the small synthesis of 2-methoxyoestriol in the absence of added ATP (Table 2).

The DPN and TPN requirements of the 7000*g* supernatant fraction (Table 6) differ in some respects from those of the homogenate. Either DPN or TPN can be employed, but the latter is more effective at higher concentrations than the former and under these conditions is as effective as DPN and TPN combined. As rat-liver mitochondria contain a transhydrogenase (Humphrey, 1957), the possibility cannot be excluded that the DPN is in fact only acting indirectly via a transhydrogenation reaction with TPN. Preincubation of the cell fraction for 10 min. at 37° indicated a partial requirement for an oxidizable substrate. The addition of nicotinamide does not influence the hydroxylase but has a pronounced effect on the amount of oestriol metabolized. Fig. 1 shows the time course of this nicotinamide-stimulated oestriol metabolism. This indicates that the lack of effect of nicotinamide on the production of 2-hydroxyoestriol is not due to an increased production of this compound compensated by an elevated destruction. ATP increases both the amount of oestriol metabolized and the formation of 2-hydroxyoestriol. This stimulation is independent of whether TPN alone or TPN plus DPN is added to the system.

When the 7000*g* supernatant fraction was separated into microsomal and 105000*g* supernatant fractions, neither fraction showed any 2-hydroxylase activity in the presence of a reduced TPN-producing system, but the further addition of either tetrahydrofolic acid or ATP to the microsomal

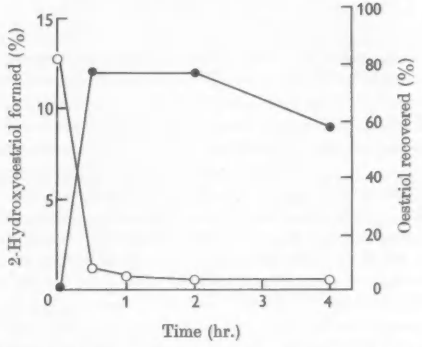

Fig. 1. Effect of varying incubation time on production of 2-hydroxyoestriol by rat-liver 7000*g* supernatant fraction. All incubations contained 1 ml. of 7000*g* supernatant fraction, 0·7 μmole of DPN, 4·5 μmoles of sodium lactate, 0·7 μmole of TPN, 4·5 μmoles of glucose 6-phosphate, 72 μmoles of nicotinamide, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 at 37°. ●, 2-Hydroxyoestriol; ○, oestriol.

Table 6. *Effect of di- and tri-phosphopyridine nucleotide and adenosine triphosphate on metabolism of oestriol by rat-liver* 7000*g* *supernatant fraction*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of 7000*g* supernatant fraction in 0·25 M-sucrose, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Final vol., 3 ml. Incubations were carried out under O_2 for 1 hr. at 37°. G-6-P, Glucose 6-phosphate.

Additions	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
0·7 μmole of DPN + 4·5 μmoles of lactate	56	5	61
1·4 μmoles of DPN + 9 μmoles of lactate	36	8	44
0·7 μmole of TPN + 4·5 μmoles of G-6-P	56	4	60
1·4 μmoles of TPN + 9 μmoles of G-6-P	32	13	45
1·4 μmoles of TPN + 9 μmoles of G-6-P + 3 μmoles of ATP	3	27	30
0·7 μmole of DPN + 0·7 μmole of TPN + 4·5 μmoles of lactate + 4·5 μmoles of G-6-P	32	16	48
0·7 μmole of DPN + 0·7 μmole of TPN + 4·5 μmoles of lactate + 4·5 μmoles of G-6-P + 72 μmoles of nicotinamide	7	15	22
0·7 μmole of DPN + 0·7 μmole of TPN + 4·5 μmoles of lactate + 4·5 μmoles of G-6-P + 3 μmoles of ATP	4	29	33

Table 7. *Effect of triphosphopyridine nucleotide, adenosine triphosphate and tetrahydrofolic acid on metabolism of oestriol by rat-liver microsomes*

Figures are given as percentages of oestriol added. All incubations contained 1 ml. of microsomal preparation in 0·25 M-sucrose, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Where stated, the following additions were also made: 1·4 μmoles of TPN, 9 μmoles of glucose 6-phosphate (G-6-P), 3 μmoles of ATP, 2·5 μmoles of tetrahydrofolic acid, 0·07 unit of G-6-P dehydrogenase. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Additions	Oestriol recovered	2-Hydroxy-oestriol formed	Total recovery
TPN + G-6-P + G-6-P dehydrogenase (oestriol added just before extraction)	81	0	81
TPN + G-6-P + G-6-P dehydrogenase	64	1	65
TPN + G-6-P + G-6-P dehydrogenase + ATP	26	4	30
Tetrahydrofolic acid + ATP	74	1	75
Tetrahydrofolic acid + ATP + TPN	—	0	—
Tetrahydrofolic acid + TPN + G-6-P + G-6-P dehydrogenase	47	6	53
Tetrahydrofolic acid + TPN + G-6-P + G-6-P dehydrogenase + ATP	29	11	40

Table 8. *Effect of a number of folic acid derivatives on the 2-hydroxylase activity of rat-liver microsomes*

All incubations contained 1 ml. of microsomal preparation in 0·25 M-sucrose, 1·4 μmoles of TPN, 9 μmoles of glucose 6-phosphate, 0·07 unit of glucose 6-phosphate dehydrogenase, 3 μmoles of ATP, 192 μmoles of potassium phosphate, pH 7·4, and 0·35 μmole (100 μg.) of oestriol in 0·025 ml. of propylene glycol. Final vol., 3 ml. Incubation was under O_2 for 1 hr. at 37°.

Additions	2-Hydroxy-oestriol formed (% of oestriol added)
2·5 μmoles of tetrahydrofolic acid	11
2·5 μmoles of dihydrofolic acid	11
2·5 μmoles of folic acid	6
2·5 μmoles of tetrahydrofolic acid (additional 2·5 μmoles added 15 min. after the start of the incubation)	11
None	4

fraction produced small amounts of 2-hydroxy-oestriol (Table 7). The best results were obtained on adding both ATP and tetrahydrofolic acid. The addition of a further 2·5 μmoles of tetrahydrofolic acid 15 min. after the start of the incubation had no effect, which suggests that the folic acid derivative is not rate-limiting. Dihydrofolic acid can replace the tetrahydro compound (Table 8) but folic acid has only a small effect.

When the microsomes were dialysed against ice-cold water for 18 hr. or were washed with 0·25 M-sucrose the 2-hydroxylase activity was lost, which suggested that some other cofactor, possibly a metal ion, is also required. This would explain the finding that only about half of the activity present in the 7000*g* supernatant could be recovered on further fractionation of this cell fraction.

Detection of other phenolic oestriol metabolites

In no case could any 6′α′-hydroxyoestriol or 6-oxo-oestriol be detected. The addition of nicotinamide to the 7000*g* supernatant fraction resulted in the detection of two new phenolic metabolites. Both were ketonic, as determined by the Girard reaction. The less-polar compound had the same mobility as 16-oxo-oestradiol-17β in both formamide–chloroform and acetic acid–water–ethylene dichloride (70:30:100, by vol.) systems. The other metabolite had the same mobility as oestriol in formamide–chloroform but it was slightly more polar than oestriol in acetic acid–water–ethylene dichloride. It was similar to 2-hydroxyoestriol in that it developed the Folin & Ciocalteu blue colour in the absence of alkali.

To characterize these compounds further, 200 μg. of oestriol was incubated under the conditions shown in Fig. 1 for 1 hr. The oestriol and 2-hydroxy-oestriol were removed by Girard separation and the two ketonic metabolites were separated by paper chromatography with formamide–chloroform for 10 hr. This method of carrying out the Girard reaction does not result in the artifactual formation of 16-oxo-oestradiol-17β from 16α-hydroxy- or 16β-hydroxy-oestrone (Layne & Marrian, 1958).

Half of the less-polar metabolite was converted into the Fast Black Salt K derivative. It had the same mobility as the Fast Black Salt K derivative of authentic 16-oxo-oestradiol-17β in ethanol–water–toluene–light petroleum (40–60°) (30:70:20:10, by vol.) (Heftmann, 1950). The other half was reduced with sodium borohydride. As judged from the chromatographic behaviour, the main reduction product was 16-*epi*oestriol plus a trace of oestriol. Sodium borohydride reduction of 16-oxo-oestradiol-17β produces 90% of 16-*epi*oestriol plus 10% of oestriol (Loke, 1958). It thus seems probable that this metabolite is 16-oxo-oestradiol-17β. Under these incubation conditions there was a 2–3% conversion of oestriol into 16-oxo-oestradiol-17β. A similar yield was obtained when a 105000*g* supernatant fraction was used.

The yield of the more-polar metabolite was too small to allow further characterization. It may

be 2-hydroxy-16-oxo-oestradiol-17β (2:3:17β-trihydroxyoestra-1:3:5-triene-16-one). Another possibility is that it is the *o*-quinone derivative of 2-hydroxyoestriol, but this seems unlikely, as such a compound would strongly absorb u.v. light in the 240 mμ region, whereas this metabolite does not. This would also seem to rule out a *p*-quinol derivative of the type discovered by Hecker & Mueller (1958).

DISCUSSION

This work substantiates the conclusion made in the preceding paper that the conversion of oestriol into 2-methoxyoestriol proceeds via 2-hydroxyoestriol.

Like other steroid hydroxylases, the 2-hydroxylase requires a reduced pyridine nucleotide. Either DPN or TPN can be utilized, although the latter is more efficient at higher concentrations. The possibility cannot altogether be excluded that DPN is acting only indirectly via a transhydrogenation reaction. It is quite possible that the 7000*g* supernatant fraction is contaminated with mitochondria, which are known to possess a transhydrogenase (Humphrey, 1957). This explanation would, however, seem unlikely, because, whereas exogenous pyridine nucleotide is required for the formation of 2-hydroxyoestriol, reasonable amounts are formed when DPN is added in the absence of TPN.

The function of the folic acid derivatives in this hydroxylation is not clear from these experiments. There are two possible explanations. The first is that a tetrahydrofolic acid derivative is the direct hydrogen donor and that the reduced pyridine nucleotide is acting only indirectly by reforming the tetrahydro compound. This is similar to the explanation given by Kaufman (1959) to account for his experiments on the conversion of phenylalanine into tyrosine. This would account for the relative effectiveness of folic acid, dihydrofolic acid and tetrahydrofolic acid in the presence of reduced TPN-producing system but does not explain the formation of 2-hydroxyoestriol in the absence of an exogenous folic acid derivative. This may be due to the presence of endogenous material in the microsomes, but as dialysis resulted in complete loss of hydroxylase activity this possibility could not be tested. The ineffectiveness of tetrahydrofolic acid in the absence of reduced-TPN-producing system could be explained by the instability of this compound in solution in the presence of oxygen. It would, however, seem to rule out the possibility that the tetrahydrofolic acid and reduced TPN are donating hydrogen to a common hydrogen-accepting cofactor.

The other possibility is that reduced TPN is the direct hydrogen donor and that the added tetrahydrofolic acid is acting by re-forming reduced TPN. This explanation does not account for the fact that excess of glucose 6-phosphate and glucose 6-phosphate dehydrogenase are present in the incubation medium and that dihydrofolic acid is as effective as tetrahydrofolic acid in stimulating the hydroxylation reaction. It would be difficult to explain the effect of a reduced-DPN-producing system by either of these hypotheses as all of the folic acid reductases so far isolated from normal mammalian tissues are TPN-specific. However, Scrimgeour & Huennekens (1960) claim to have detected a DPN-specific reductase in Ehrlich ascites-tumour cells.

If a folic acid derivative is required for 2-hydroxylation it would provide an interesting link between the observation that folic acid is needed for the oestrogen-stimulated growth of chicken oviduct (Hertz, 1948) and the fact that 2- and 4-hydroxyoestradiol-17β are the only compounds so far tested which have an effect on uterine protein metabolism *in vitro* (Mueller, 1955).

The exact function of ATP in hydroxylation reactions is not certain. Hayano & Dorfman (1954) found that the DPN and ATP, previously thought to be required for 11β-hydroxylation, could be replaced by TPN, which indicated that the ATP was converting the DPN into TPN. Similar results have been obtained with the adrenal-microsomal 21-hydroxylase (Ryan & Engel, 1957). ATP stimulates the hydroxylation of acetanilide by liver microsomes in the presence of TPN plus a reduced TPN-producing system but had no effect when reduced TPN alone was added (Mitoma, Posner, Reitz & Udenfriend, 1956). It is difficult to reconcile the results given in the present paper with these explanations. If ATP is acting by phosphorylating DPN it should not stimulate the microsomal 2-hydroxylase in the absence of DPN. Also, in the experiments with the 7000*g* supernatant fraction, the ATP activation is independent of whether TPN alone or TPN plus DPN is added to the incubation medium. Under the conditions used in these experiments, the reduction of TPN is very rapid in the absence of ATP so it would seem unlikely that it is stimulating the production of reduced TPN. The fact that the 2-hydroxylating system is not affected by nicotinamide rules against pyridine nucleotide breakdown being a rate-limiting reaction, but the possibility cannot be eliminated that the ATP is stabilizing the pyridine nucleotides by some other mechanism. It is noteworthy that Kornberg (1950) has described an enzyme which catalyses the following reaction:

$$\text{ATP} + \text{nicotinamide mononucleotide} \rightleftharpoons \text{DPN} + \text{pyrophosphate}.$$

The ATP could also be stimulating the reaction by activating a folic acid compound. Greenberg & Jaenicke (1957) and Whiteley, Osborn & Huennekens (1958) have postulated a phosphorylated

tetrahydrofolic acid compound as an intermediate in the activation of formic acid.

The loss of activity on washing or dialysing the microsome fraction suggests that another cofactor, possibly a metal ion, is required.

As far as can be judged from the experiments with crude homogenates, the 2-hydroxylase does not appear to be very specific.

The small formation of 2-methoxyoestriol in the absence of ATP was rather unexpected. In other cases of *O*-methylation (Axelrod & Tomchick, 1958; Pellerin & D'Ionio, 1958) the methyl group is derived from *S*-adenosylmethionine and it seems likely that the *o*-methyl group of 2-methoxyoestriol has a similar origin. The small amount of 2-methoxyoestriol formed in the absence of ATP is presumably due to the presence of endogenous ATP or an ATP-synthesizing system. The finding that rabbit-liver slices produce less 2-methoxyoestriol but more 2-hydroxyoestriol than do rat-liver slices (King, 1961) suggests that the methylase studied in these experiments is the same as that studied by Axelrod & Tomchick (1958), as they found that rabbit liver had a much lower *O*-methylating activity than rat liver.

The formation of 16-oxo-oestradiol-17β from oestriol in the liver is very small compared with that in the kidney (King, 1960).

The absence of 6′α′-hydroxyoestriol and 6-oxo-oestriol was rather surprising, especially as the microsomal system used in these experiments is almost identical with that with which Mueller & Rumney (1957) obtained a high yield of 6′α′-hydroxyoestradiol-17β from oestradiol-17β. Moreover, Breuer, Knuppen & Schriefers (1960) have recently reported the conversion of oestriol into 6-hydroxyoestriol by rat-liver slices. In view of the large amounts of oestriol which cannot be accounted for, it is possible that small amounts of these compounds are formed but are further metabolized to undetected products. In this respect it is interesting that no 6′α′-hydroxyoestriol or 6-oxo-oestriol could be detected in human pregnancy urine (G. F. Marrian & R. J. B. King, unpublished results).

Graubard & Pincus (1942) showed that oestrogens could be oxidized by a number of plant phenolases and this work has recently been extended by Jellinck (1960). The virtual absence of oestriol metabolism in the absence of DPN and TPN suggests that such reactions do not occur in rat liver.

SUMMARY

1. The quantitative production of 2-hydroxyoestriol and 2-methoxyoestriol has been studied with rat-liver preparations. The 2-hydroxylase requires either a reduced diphosphopyridine nucleotide- or reduced triphosphopyridine nucleotide-producing system and possibly a folic acid derivative. Adenosine triphosphate has a stimulating effect. The 2-hydroxylase is localized in the microsomal fraction.

2. The 2-hydroxylase activity is absent in homogenates of kidney, ovary and uterus. There is no sex difference in the rat-liver activity.

3. The *O*-methylating system requires magnesium ions, adenosine triphosphate and L-methionine. Evidence is presented that the *o*-methylase may be the same enzyme as that studied by Axelrod & Tomchick (1958).

4. Preliminary evidence suggests that oestrone, 17α-ethynyloestradiol-17β, stilboestrol and possibly oestradiol-17β can be methoxylated in the position *ortho* to an existing phenolic group.

5. 16-Oxo-oestradiol-17β and another unknown compound have been detected as minor metabolites of oestriol. The oestriol-16-hydroxy dehydrogenase is localized in the 105000*g* supernatant fraction.

6. The relationship of reduced pyridine nucleotide, folic acid and adenosine triphosphate to hydroxylation reactions has been discussed.

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Effects of Metal Ions on the Utilization of Glucose and on the Influence of Insulin on it by the Isolated Rat Diaphragm

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Insulin causes an increased uptake of glucose by the isolated rat diaphragm (Gemmill, 1940), although the mechanism by which insulin exerts its effect has not yet been explained. On the basis of the evidence presented by Levine & Goldstein (1955), Park, Bornstein & Post (1955), Park & Johnson (1955), Park, Johnson, Wright & Batsel (1957) and others, it is, however, now generally agreed that insulin, both *in vivo* and *in vitro*, exerts its effect by increasing the rate of entry of glucose from the extracellular fluid to the interior of the cell, presumably by some effect on the cell membrane. Nothing, however, is so far known regarding the nature of the effect of insulin on the cell membrane.

This paper describes the results of experiments on the effects of physiologically important cations, and of certain other related ions, on the uptake of glucose and on the influence of insulin on it by the rat diaphragm *in vitro*. It is well known that these ions, in addition to their functions of osmotic regulation of tissue fluids and of activation of various enzymes, play essential roles in the preservation of integrity, and the regulation of permeability properties, of cell membranes. Preliminary reports of some of the experiments described in this paper have been published (Bhattacharya, 1959*a*, *b*).

MATERIALS AND METHODS

Incubation media. The standard incubation medium was the bicarbonate buffer of Gey & Gey (1936). Various other incubation mixtures were used. These latter mixtures generally consisted of a suitable buffer in iso-osmotic sucrose (0·25 M) or in a mixture of iso-osmotic sucrose and iso-osmotic solutions of the chlorides of certain univalent and bivalent metals. 0·154 M-Solutions of the chlorides of the univalent metals and 0·11 M-solutions of the chlorides of bivalent metals were considered to be iso-osmotic. In most instances the pH of the medium was adjusted to 7·4. The buffers used and their concentrations in the medium were usually as follows: $LiHCO_3$, $NaHCO_3$ and $KHCO_3$, 0·02 M; Na_2HPO_4, 0·01 M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0·025 M. Glucose when present was added to give a final concentration of about 250 mg./100 ml.

Insulin. Crystalline insulin (Wellcome Foundation or Boots Pure Drug Co. Ltd.) was dissolved in 0·033 N-HCl to give a concentration of 10 units/ml. This stock insulin was added to the incubation medium to give the required concentration just before each experiment. The final insulin concentration was usually about 0·1 unit/ml.

^{131}I-*marked insulin.* This was prepared according to the method of Boursnell, Coombs & Rizk (1953) as modified by Boursnell (1958). The final stock preparation was obtained in 0·05 M-Na_2HPO_4 buffer, pH 7·4. The insulin concentration was about 0·5 mg./ml. No inactivation of the insulin, as measured by its effect on increased uptake of glucose by the isolated rat diaphragm, could be demonstrated.

Animals. Diaphragm muscle was obtained from female albino Wistar rats (wt. 100–150 g.) which had been starved 20–24 hr. before use.

Preparation of cut diaphragm. The rat was killed by decapitation and bled. The diaphragm was then removed and put into a beaker containing freshly gassed buffer. After the required number (usually six) of diaphragms had been obtained, each diaphragm was gently blotted and cut into halves, and each half was transferred to a small conical flask containing 1 ml. of the medium. One-half of a diaphragm served as the control and the other was used to determine the effect of insulin or of other treatment. The flasks were then gassed with the required gas mixture (see Table 1), sealed with rubber stoppers and incubated at 38°

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in a Dubnoff shaker usually for 60 or 90 min. After incubation the hemidiaphragms were taken out, blotted and weighed on a torsion balance, and the medium was analysed.

Preparation of intact diaphragm. The rat was killed by decapitation and bled, and the intact diaphragm was removed as described by Kipnis & Cori (1957). The diaphragm was washed in freshly gassed medium and then slid into a 50 ml. conical flask containing 15 ml. of the medium. The flasks were gassed with the required gas mixture, sealed with rubber stoppers and incubated at 38° in a Dubnoff shaker for 1 hr. After incubation the tissue was taken out of the flask and the diaphragm (excepting the posterior portion) excised from the rib cage. It was then blotted, weighed on a torsion balance and analysed. The medium in the flask was also analysed.

Measurement of pH. This was done with the glass electrode.

Glucose uptake. This was determined by measuring the disappearance of glucose from the incubation medium. Samples of the medium were deproteinized with $BaSO_4$ (Somogyi, 1945), and the sugar content of the protein-free filtrate was determined either by the Somogyi (1945) modification of Nelson's method or by the method of Hagedorn & Jensen (1923), as found convenient.

Xylose. For estimating xylose in the diaphragm after incubation, the muscle was rapidly made into a paste with 20 vol. of water by grinding with sand in a mortar and deproteinizing with $BaSO_4$ (Somogyi, 1945). The protein-free filtrate was analysed for xylose by the *p*-bromoaniline method of Roe & Rice (1948). The xylose content of the medium was also similarly determined.

Xylose space. The space was calculated as follows:

$$\text{Space (ml./100 g.)} = \frac{\text{muscle content (g./100 g. of wet muscle)}}{\text{medium concentration (g./ml.)}}.$$

Measurement of binding of insulin by diaphragm. Diaphragms were incubated for 0·5 hr. at 38° under the desired conditions in the presence of ^{131}I-marked insulin. One hemidiaphragm was suspended in 1 ml. of the medium containing about 0·1 unit of insulin. After incubation the hemidiaphragms were removed, washed in appropriate media for 10 min. with three changes, blotted, weighed and spread on aluminium planchets. They were then dried under an infrared lamp and counted with a thin end-window Geiger–Müller tube. Individual hemidiaphragms gave 400–1100 counts/min. and at least 1000 counts were recorded for each sample.

Statistical treatment of results. In most cases experiments were done in groups of six and the significance of the differences between the means was assessed on the basis of Student's *t* test. A value of *P* (probability that the difference was due to chance) less than 0·05 was considered to be significant.

Table 1. *Influence of insulin on glucose uptake by rat diaphragm from iso-osmotic sucrose in the presence or absence of small amounts of metal ions*

Hemidiaphragms were incubated in iso-osmotic sucrose (1 hemidiaphragm/ml.) buffered with $NaHCO_3$, $KHCO_3$ or $LiHCO_3$ (0·02 M), Na_2HPO_4 (0·01 M) or tris (0·025 M). The gas phase was $O_2 + CO_2$ (95:5) with the bicarbonate buffers, and O_2 with phosphate and tris buffers. Incubation was for 1·5 hr. at 38°.

		Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)		
Medium	Buffer	Without insulin	With insulin (0·1 unit/ml.)	*P*
Gey's salt mixture	$NaHCO_3$	3·16 ± 0·14	7·67 ± 0·44	< 0·001
Sucrose	$NaHCO_3$	4·15 ± 0·15	4·18 ± 0·21	> 0·05
Sucrose	$KHCO_3$	4·11 ± 0·28	4·64 ± 0·26	< 0·01
Sucrose	$LiHCO_3$	6·49 ± 0·20	6·61 ± 0·21	> 0·05
Sucrose	Na_2HPO_4	4·58 ± 0·19	4·87 ± 0·15	> 0·05
Sucrose	Tris	4·43 ± 0·22	4·68 ± 0·15	> 0·05

Table 2. *Effect of alkali-metal ions on the influence of insulin in causing increased uptake of glucose by rat diaphragm*

Experimental details were the same as under Table 1. Iso-osmotic solutions of sucrose and of the different metal chlorides were mixed in various proportions to give the desired concentration of the salts in the medium.

		Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)		
Buffer	Electrolyte added and its concn. in the medium (mM)	Without insulin	With insulin (0·1 unit/ml.)	*P*
$NaHCO_3$	NaCl (32)	4·45 ± 0·20	6·95 ± 0·17	< 0·001
$NaHCO_3$	NaCl (62)	5·16 ± 0·28	8·04 ± 0·40	< 0·001
Na_2HPO_4	NaCl (77)	3·84 ± 0·30	6·12 ± 0·27	< 0·001
Tris	NaCl (77)	4·46 ± 0·12	7·18 ± 0·06	< 0·001
$KHCO_3$	KCl (32)	2·99 ± 0·25	5·59 ± 0·18	< 0·001
$KHCO_3$	KCl (64)	2·82 ± 0·22	4·81 ± 0·17	< 0·001
$LiHCO_3$	LiCl (64)	7·95 ± 0·31	9·10 ± 0·31	< 0·001
Tris	LiCl (77)	6·80 ± 0·24	7·93 ± 0·40	< 0·001
Tris	RbCl (51)	3·24 ± 0·21	5·52 ± 0·33	< 0·001
Tris	CsCl (51)	3·30 ± 0·13	6·33 ± 0·37	< 0·001

RESULTS

Influence of moderate amounts of alkali-metal ions on the uptake of glucose and on the influence of insulin on it by rat diaphragm. Diaphragm incubated in iso-osmotic sucrose (0·25 M), buffered at pH 7·4 with tris (0·025 M), Na_2HPO_4 (0·01 M) or $NaHCO_3$ or $KHCO_3$ (0·02 M), removed glucose from the medium in amounts comparable with those removed from the bicarbonate buffer of Gey & Gey (1936). Insulin, however, had little or no effect on the uptake of glucose under such conditions (Table 1). With $LiHCO_3$ (0·02 M) as the buffer, there was also little effect of insulin, although in this case the basal uptake of glucose was considerably higher. The glucose uptake given in this Table and elsewhere represents an extent of uptake rather than a rate. It has been shown (see Khral, 1951) that the basal uptake is highest if measured for the first 0·5 hr. of incubation, less if measured for a 1 hr. period and still less for a 2 hr. period of measurement. Thus the uptake does not proceed linearly over the usual period (1–2 hr.) of measurement. The observed effects with insulin and ions reported in this paper also represent an extent rather than a rate.

Table 3. *Comparison of glucose uptake by rat diaphragm in media containing equivalent amounts of the alkali-metal ions*

Experiments were done in pairs by using hemidiaphragms from the same rat and then comparing two of the metal chlorides in turn. The experimental details were otherwise the same as in Table 2.

Metal ion and its concn. in the medium (mM)	Buffer	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)	P
Li^+ (64)	Bicarbonate	7·48 ± 0·26	< 0·05
Na^+ (64)	Bicarbonate	5·83 ± 0·43	
Na^+ (64)	Bicarbonate	5·74 ± 0·37	< 0·01
K^+ (64)	Bicarbonate	3·08 ± 0·18	
Li^+ (77)	Tris	7·37 ± 0·45	< 0·001
Na^+ (77)	Tris	4·26 ± 0·27	
Na^+ (77)	Tris	4·14 ± 0·27	< 0·01
K^+ (77)	Tris	2·55 ± 0·21	
K^+ (77)	Tris	2·30 ± 0·14	> 0·05
Rb^+ (77)	Tris	2·44 ± 0·16	
Rb^+ (77)	Tris	2·05 ± 0·16	< 0·02
Cs^+ (77)	Tris	1·56 ± 0·10	
Li^+ (20)	Bicarbonate	5·40 ± 0·15	< 0·001
Na^+ (20)	Bicarbonate	3·73 ± 0·15	
Na^+ (20)	Bicarbonate	3·69 ± 0·13	< 0·001
K^+ (20)	Bicarbonate	2·97 ± 0·14	

Table 4. *Comparison of glucose uptake by rat diaphragm which had been previously incubated in media containing equivalent amounts of* Li^+, Na^+ *and* K^+ *ions*

Hemidiaphragms were incubated for 0·5 hr. at 38° in iso-osmotic sucrose (1 hemidiaphragm/ml. of the medium without any added glucose) containing $LiHCO_3$, $NaHCO_3$ or $KHCO_3$ (0·02 M). The gas phase was $O_2 + CO_2$ (95:5). The hemidiaphragms were then incubated for 1 hr. at 38° in Gey's buffer containing about 250 mg. of glucose/100 ml. and the uptake of glucose was measured.

Preincubation	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)	P
$LiHCO_3$	6·22 ± 0·39	< 0·01
$NaHCO_3$	4·40 ± 0·14	
$KHCO_3$	3·07 ± 0·16	< 0·01

Addition of moderate amounts of the chlorides of sodium or potassium or of the other related univalent metals, lithium, rubidium and caesium, to the inactive sucrose medium, however, resulted in the appearance of an insulin effect (Table 2). The response to insulin in media containing Li^+ ions was small. This apparently resulted from the high basal uptake of glucose from such media (Table 2). The basal uptake of glucose in fact varied in a regular way in the presence of equivalent amounts of the different metal ions in the medium. A comparison of the glucose uptake by the diaphragm in the presence of equivalent amounts of the alkali-metal ions in the medium is given in Table 3, which shows that the uptake was highest with Li^+ ions and lowest with Cs^+ ions and followed the order $Li^+ > Na^+ > K^+ = Rb^+ > Cs^+$. This influence of the alkali-metal ions in regulating the basal glucose uptake by the rat diaphragm was exerted even when the diaphragms were preincubated (without glucose) for a short period in media containing the different ions, the final incubation during which the uptake of glucose was measured being carried out in Gey's buffer (Table 4).

Influence of small amounts of bivalent metal ions on the insulin-sensitivity of the rat diaphragm. Low concentrations of Mg^{2+} ions promoted increased uptake of glucose due to insulin from the inactive sucrose medium buffered with $NaHCO_3$ (0·02 M) or Na_2HPO_4 (0·01 M) (Table 5). Mg^{2+} ions also had a tendency to increase the basal uptake itself. Of the other bivalent ions, Ca^{2+} and Zn^{2+} ions had clearly no effect. Mn^{2+} ions might have had some effect, but it was not very clear-cut. Ca^{2+} ions in fact counteracted the influence of Mg^{2+} ions in promoting insulin response to the tissue (Table 6). This inhibitory effect of Ca^{2+} ions could be overcome by increasing the concentration of Mg^{2+} ions in the medium.

Also Ca^{2+} ions had a strong depressing effect on the basal glucose uptake itself (Table 6).

Table 5. *Effects of the addition of low concentration of bivalent metal ions on the influence of insulin in causing increased uptake of glucose by rat diaphragm from inactive sucrose media*

Hemidiaphragms were incubated in iso-osmotic sucrose (1 hemidiaphragm/ml.) buffered with $NaHCO_3$ (0·02M) or Na_2HPO_4 (0·01M) for 1 hr. at 38° under the appropriate gas phase as given in Table 1.

	Addition		Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)	
Buffer	Salt	Concn. (mM)	Without insulin	With insulin (0·1 unit/ml.)
$NaHCO_3$	None	—	4·16 ± 0·17	4·86 ± 0·14
$NaHCO_3$	$MgCl_2$	0·2	4·40 ± 0·10	6·12 ± 0·11
$NaHCO_3$	$MgCl_2$	1·0	5·53 ± 0·31	7·97 ± 0·12
$NaHCO_3$	$CaCl_2$	0·2	4·84 ± 0·20	5·00 ± 0·18
$NaHCO_3$	$CaCl_2$	1·0	3·36 ± 0·16	3·92 ± 0·21
$NaHCO_3$	$MnCl_2$	0·2	4·85 ± 0·18	5·93 ± 0·14
$NaHCO_3$	$MnCl_2$	1·0	4·82 ± 0·26	6·15 ± 0·18
$NaHCO_3$	$ZnSO_4$	0·2	4·25 ± 0·18	4·50 ± 0·24
$NaHCO_3$	$ZnSO_4$	1·0	4·03 ± 0·15	4·45 ± 0·24
Na_2HPO_4	None	—	4·25 ± 0·13	4·56 ± 0·17
Na_2HPO_4	$MgCl_2$	1·0	5·01 ± 0·71	8·50 ± 0·67

Table 6. *Antagonism between* Ca^{2+} *and* Mg^{2+} *ions with respect to increased uptake of glucose due to insulin by the rat diaphragm* in vitro

The basal incubation medium was iso-osmotic sucrose buffered with $NaHCO_3$ (0·02M). The gas phase was $O_2 + CO_2$ (95:5). Incubation was for 1 hr. at 38°.

	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)	
Additions	Without insulin	With insulin (0·1 unit/ml.)
1 mM-$MgCl_2$	5·94 ± 0·22	8·22 ± 0·16
1 mM-$MgCl_2$ + 5 mM-$CaCl_2$	3·40 ± 0·22	5·35 ± 0·30
5 mM-$MgCl_2$ + 5 mM-$CaCl_2$	4·46 ± 0·30	7·69 ± 0·32

Table 7. *Influence of* Na^+ *and* Mg^{2+} *ions on the increased uptake of glucose due to insulin by rat diaphragm from an iso-osmotic sucrose medium buffered to* pH 7·0 *with* 5·9 mM-*sodium bicarbonate*

Hemidiaphragms were incubated in the above medium (1 hemidiaphragm/ml. of medium) for 1 hr. at 38° in $O_2 + CO_2$ (95:5).

	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.)	
Additions	Without insulin	With insulin (0·1 unit/ml.)
14 mM-NaCl	3·85 ± 0·23	4·97 ± 0·16
1 mM-$MgCl_2$	4·90 ± 0·23	5·39 ± 0·29
14 mM-NaCl + 1 mM-$MgCl_2$	3·79 ± 0·08	6·30 ± 0·24

Although the presence of 1 mM-Mg^{2+} considerably increased the uptake of glucose due to insulin from an iso-osmotic sucrose medium containing 0·02M-$NaHCO_3$ (Table 5), there was no response to insulin in a medium containing 5·9 mM-$NaHCO_3$, pH 7·0, in $O_2 + CO_2$ (95:5), even in the presence of the same concentration of Mg^{2+} ions (Table 7). Addition of NaCl to this medium so as to make the concentration of Na^+ ions about 0·02M led to the appearance of an insulin effect. Addition of NaCl (about 0·02M) alone (in the absence of added Mg^{2+} ions) produced only a small response to insulin.

Influence of ethylenediaminetetra-acetic acid on the increased uptake of glucose due to insulin from sucrose–sodium chloride medium. Addition of ethylenediaminetetra-acetic acid (EDTA) to a concentration of 2 mM largely abolished the increased uptake of glucose due to insulin from a sucrose medium containing 0·077M-NaCl and buffered with tris (0·025M) (Table 8). This inhibitory effect of EDTA was fully counteracted by the presence of 2 mM-Mg^{2+} in the medium.

Role of Mg^{2+} *ions in maintaining insulin-sensitivity of the isolated rat diaphragm.* The results of the foregoing experiments showed that a small amount of Mg^{2+} ions was critically important for insulin-responsiveness of the rat diaphragm. Some idea of the nature of this action of Mg^{2+} ions was obtained in a series of experiments in which diaphragms were first incubated for 0·5 hr. in media with or without added Mg^{2+} ions and without any added glucose. The effect of this preincubation on the insulin-sensitivity of the tissue, as measured by increased uptake of glucose in the presence of insulin from Gey's buffer, was then tested. The results showed that insulin caused the usual increase in the uptake of glucose by diaphragm which had been preincubated for 0·5 hr. in Gey's buffer either at room temperature or at 38°. The ability of the tissue to respond to insulin was also left unimpaired when it was preincubated for 0·5 hr. at room temperature in iso-osmotic sucrose buffered with $NaHCO_3$ (0·02M). Preincubation for 0·5 hr. at 38° in the sucrose–bicarbonate buffer,

however, caused a marked loss of the insulin-sensitivity of the diaphragm; this did not occur in the presence of a low concentration of Mg^{2+} ions (1 mM). Ca^{2+} ions in suitable concentrations counteracted the protective effect of Mg^{2+} ions, and the action of Ca^{2+} could be overcome by increasing the concentration of Mg^{2+} ions in the medium. Mn^{2+} ions could not protect the tissue against loss of insulin-sensitivity due to the preincubation.

Stadie, Haugaard & Vaughan (1952, 1953) showed that rat diaphragm which had been previously incubated with insulin in appropriate media showed increased glycogen synthesis during subsequent incubation without any added insulin. The influence of ions in this phenomenon was studied. It was observed that a hemidiaphragm which had been preincubated for 0·5 hr. in the presence of 0·1 unit of insulin/ml. in iso-osmotic sucrose buffered with either $NaHCO_3$ (0·02 M) or tris (0·025 M) showed increased uptake of glucose during subsequent incubation in Gey's buffer. The increment was comparable with those obtained when the preincubation was done in Gey's buffer itself. EDTA (5 mM) in the sucrose medium during the preincubation, however, depressed the increased uptake of glucose considerably during subsequent incubation. This inhibitory effect of EDTA could again be fully counteracted by Mg^{2+} ions (5 mM) in the preincubation medium.

Effect of anaerobiosis on the increased uptake of glucose due to insulin. The experiments so far reported were all carried out under aerobic conditions. When some of them were repeated under anaerobic conditions (Table 9), there was little or no increased uptake of glucose due to insulin even when the medium contained the required ions in sufficient amounts. With bicarbonate buffer, the basal uptake of glucose, however, tended to be higher under anaerobic conditions than under aerobic conditions. This was particularly true for the sucrose–NaCl medium (Expt. no. 1, Table 9). Anaerobiosis has a similar effect on the basal uptake of glucose by rat diaphragm from Gey's bicarbonate buffer (Randle, 1956).

In a few preincubation experiments under anaerobic conditions it was observed that preincubation of the diaphragm with insulin in Gey's medium (without glucose) under anaerobic conditions did not lead to any increased uptake of glucose during subsequent incubation either under anaerobic or under aerobic conditions. Preincubation under anaerobic conditions also led to the abolition of the increased uptake of glucose under aerobic conditions.

Influence of lack of ions and of oxygen on the binding of insulin by rat diaphragm. Incubation of diaphragm at 38° under anaerobic conditions in Gey's buffer or under aerobic conditions in iso-osmotic sucrose buffered with 0·02 M-$NaHCO_3$ led to a loss of insulin-sensitivity of the tissue. The effect of such preincubations on the binding of insulin by the tissue (Stadie *et al.* 1952, 1953) was studied with ^{131}I-marked insulin (Table 10). It was observed that incubation in the sucrose–bicarbonate medium under aerobic conditions largely increased the amount of insulin bound by the tissue as compared

Table 8. *Influence of ethylenediaminetetra-acetic acid on the increased uptake of glucose due to insulin from sucrose–sodium chloride medium buffered with tris*

Hemidiaphragms were incubated in an iso-osmotic sucrose–NaCl mixture in which the concentration of NaCl was 0·077 M and which was buffered with tris (0·025 M) at pH 7·4. Incubation was for 1 hr. at 38° in O_2.

Additions	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.) Without insulin	With insulin (0·1 unit/ml.)
None	4·46 ± 0·12	7·18 ± 0·06
0·5 mM-EDTA	3·00 ± 0·17	7·17 ± 0·35
2 mM-EDTA	4·07 ± 0·24	5·82 ± 0·22
2 mM-EDTA + 2 mM-$MgCl_2$	3·58 ± 0·24	7·86 ± 0·22

Table 9. *Influence of insulin on glucose uptake by rat diaphragm incubated in iso-osmotic sucrose containing moderate amounts of Na^+ or K^+ ions under anaerobic conditions*

Hemidiaphragms were incubated in mixtures of iso-osmotic sucrose and iso-osmotic NaCl or KCl buffered with bicarbonate (0·02 M), Na_2HPO_4 (0·01 M) or tris (0·025 M) to pH 7·4. One hemidiaphragm was suspended in 1 ml. of the medium. Incubation was carried out under anaerobic conditions, $N_2 + CO_2$ (95:5) with bicarbonate buffers and N_2 with phosphate or tris buffer, for 1·5 hr. at 38°.

Expt. no.	Metal ion and its concn. in the medium (mM)	Buffer	Glucose uptake (mean ± S.E.M.) (mg./g. of wet tissue/hr.) Without insulin	With insulin (0·1 unit/ml.)	P
1	Na^+ (64)	$NaHCO_3$	7·91 ± 0·22	8·39 ± 0·25	> 0·05
2	Na^+ (77)	$NaHPO_4$	3·32 ± 0·12	3·52 ± 0·12	> 0·05
3	Na^+ (77)	Tris	2·52 ± 0·09	2·81 ± 0·07	< 0·02
4	K^+ (64)	$KHCO_3$	3·93 ± 0·25	4·40 ± 0·21	< 0·05

Table 10. *Binding of insulin by rat diaphragm in iso-osmotic sucrose buffered with sodium bicarbonate (0·02 M) under aerobic conditions and from Gey's buffer both under aerobic and under anaerobic conditions*

One hemidiaphragm of a rat was incubated in 1 ml. of Gey's buffer containing 0·1 unit of ^{131}I-marked insulin /ml. under aerobic conditions at 38° for 0·5 hr. The corresponding hemidiaphragm was similarly incubated in iso-osmotic sucrose buffered with $NaHCO_3$ (0·02 M) under aerobic conditions, and the amount of insulin bound was determined.

Incubation	Amount of insulin bound (mean ± S.E.M.) (counts/min./g. of wet tissue)	*P*
In Gey's medium, aerobic	4 952 ± 188	< 0·001
In iso-osmotic sucrose–$NaHCO_3$ medium, aerobic	12 228 ± 712	
In Gey's medium, anaerobic	4 879 ± 207	> 0·05
In Gey's medium, anaerobic	4 121 ± 205	

Table 11. *Xylose-spaces of diaphragms incubated in the presence of* Li^+, Na^+ *and* K^+ *ions* (77 *mM*)

Intact diaphragms were incubated in mixtures of iso-osmotic sucrose, and iso-osmotic chlorides and bicarbonates of the alkali metals so that the bicarbonate concentration was 0·02 M. The incubation medium contained 150 mg. of D-xylose/100 ml. The gas phase was $O_2 + CO_2$ (95:5). Incubation was for 1 hr. at 38°.

Metal ion	Xylose-space (mean ± S.E.M.) (ml. of water/100 g. of wet tissue)	*P*
Li^+	56 ± 2·3	< 0·01
Na^+	47 ± 0·9	< 0·01
K^+	35 ± 2·5	

with that bound from Gey's buffer under similar conditions. There was, however, no difference in the amounts of insulin bound under aerobic or anaerobic conditions from Gey's buffer.

Xylose-spaces of diaphragms incubated in the presence of equivalent amounts of Li^+, Na^+ and K^+ *ions.* Table 11 gives a comparison of the xylose-spaces of intact diaphragms incubated in bicarbonate buffers containing equivalent amounts of Li^+, Na^+ or K^+ ions. The results show that the xylose-space was highest in the medium containing Li^+ ions and lowest in the medium containing K^+ ions. The xylose-spaces of the diaphragm in the different media were thus directly proportional to the glucose uptake by the diaphragm from such media (Table 3).

DISCUSSION

The experiments described in this paper show that insulin increases the uptake of glucose by the isolated rat diaphragm only in the presence of certain metal ions. Moderate concentrations of any of the different alkali-metal ions are effective in this respect but, of the bivalent-metal ions tested, only Mg^{2+} ions are effective.

The Mg^{2+} ion is of particular interest in this respect. The presence of a low concentration of Mg^{2+} ions enabled the diaphragm to respond to insulin under ionic conditions in which otherwise there was little or no response (Table 5). The results in Tables 7 and 8, however, indicate that two ions, small concentrations of Mg^{2+} ions and moderate concentrations of Na^+ ions (or of some other alkali-metal ions), are possibly required for a proper insulin action. A medium containing 0·02 M-sodium bicarbonate plus 1 mM-magnesium chloride, pH 7·4, showed considerable insulin effect, but there was little effect in a medium containing 5·9 mM-sodium bicarbonate and 1 mM-magnesium chloride, pH 7·0, although raising the Na^+ ion concentration to 0·02 M re-established the insulin stimulation (Table 7). The presence of 2 mM-EDTA largely abolished the increased uptake of glucose due to insulin from a sucrose medium containing only 0·077 M-sodium chloride (buffered with tris) and this inhibitory effect was overcome by the addition of 2 mM-magnesium chloride (Table 8). This indicates that the effect of large amounts of Na^+ ions (and possibly of the other alkali-metal ions) in promoting insulin-sensitivity to the tissue is perhaps associated with a simultaneous sparing action of the alkali-metal ions on the magnesium present in the tissue *in situ*.

The nature of the action of Mg^{2+} ions in maintaining insulin-sensitivity of the tissue is not clear from the present data. It seems, however, that Mg^{2+} ions possibly act by controlling the activity of some enzyme in the system. This is indicated by the antagonism between Mg^{2+} and Ca^{2+} ions with respect to the insulin-sensitivity of the tissue, and also by the sharp loss of insulin-sensitivity at 38° but not at room temperature when the tissue is incubated in iso-osmotic sucrose–sodium bicarbonate (0·02 M) buffer. It is also indicated by the observation previously reported (Bhattacharya, 1959*b*) that when diaphragms are put into iso-osmotic sucrose buffered with 0·02 M-sodium bicarbonate (and also with 0·01 M-disodium hydrogen phosphate) they begin to contract vigorously after a few minutes and that addition of Mg^{2+} ions in concentrations which preserve the insulin-sensitivity of the tissue also stops the contraction. The bivalent metal ions, Ca^{2+} or Zn^{2+}, which are ineffective in maintaining the tissue sensitive to insulin, are also ineffective in stopping the contraction.

The loss of insulin-sensitivity of the diaphragm in the inactive sucrose medium at 38° is associated

with an increased binding of insulin by the tissue. Assuming that the binding of insulin takes place on cell surfaces, as postulated by Stadie (1954, 1956), the increased binding of insulin by the diaphragm at 38° in the inactive sucrose medium possibly indicates some profound change in the nature of the cell membranes. It is this change in the nature of the cell membranes that is possibly responsible for the loss of sensitivity to insulin of the tissue. When diaphragms are incubated anaerobically in Gey's buffer, they lose their sensitivity to insulin. However, there is no difference in the amounts of insulin bound by the diaphragm due to incubation under aerobic or anaerobic conditions from Gey's buffer.

The experiments described in this paper stress the importance of an aerobic environment for insulin action. Insulin causes increased uptake of glucose only under aerobic conditions. This has been demonstrated by a number of workers (Walaas & Walaas, 1952; Demis & Rothstein, 1954). The dependence of insulin action on an aerobic environment has had various explanations. Demis & Rothstein (1954) suggest that this indicates that the action of insulin is dependent on the presence of some cellular constituent which is present in adequate amounts only under aerobic conditions. Randle & Smith (1958), on the basis of their experiments on the effects of anaerobiosis and of certain metabolic inhibitors including 2:4-dinitrophenol on glucose uptake by the isolated diaphragm, consider that oxidative phosphorylation is involved in the process, although possibly in an indirect way. The present author, however, believes that oxidative phosphorylation is not involved in the phenomenon. The dependence of insulin action on an aerobic environment does not necessarily implicate oxidative phosphorylation. Assuming that insulin interacts with certain constituents (possibly protein in nature) of the cell surface, thus changing their nature and the permeability properties of the surface, the presence of oxygen might be necessary merely for the interaction to take place in the right way. Indeed, it is not clear to the author why some enzymic or transport mechanism must necessarily be invoked (as is the present tendency) in attempts to explain insulin action. Many important biological reactions, such as antigen–antibody reactions, are only protein–protein reactions without the involvement of any enzyme. There seems to be no reason why insulin action cannot be thought to be one of such biological reactions.

The alkali metals affected the basal uptake of glucose by the diaphragm in a regular way, apparent both in the concentrations at which they sustained the insulin-sensitivity of the tissue and in the concentrations at which they failed to do so, and the tissue lost permanently its ability to respond to insulin. This latter phenomenon shows that the ways in which the metal ions and insulin influence the glucose uptake are possibly separate and distinct from each other, one operating independently of the other. Insulin possibly acts by affecting certain magnesium-dependent sites on the cell surface. The metal ions, on the other hand, possibly act by influencing other sites. There are, however, interesting similarities between the action of insulin and the action of the alkali-metal ions in regulating the uptake of glucose by the diaphragm. The xylose-spaces of the diaphragm in media containing equivalent amounts of the alkali-metal ions (Table 11) are directly proportional to the glucose uptake by the diaphragm from such media (Table 3). Thus the varied uptake of glucose under the influence of the different alkali-metal ions is possibly brought about by a control of the rate of entry of the sugar from the extracellular fluid to the interior of the cell. This is the way in which insulin also is now considered to increase the utilization of glucose by the muscle. Further, the ions varied the basal uptake in a regular way even when the diaphragms were preincubated for a short period in media containing the different ions and subsequently incubated in Gey's buffer with glucose (Table 4). This effect of preincubation with the metal ions is again strikingly similar to the effect of preincubation of the diaphragm with insulin on increased uptake of glucose during subsequent incubation without any added insulin, first observed by Stadie *et al.* (1952, 1953) and confirmed in the present work. Stadie (1954, 1956) has interpreted this result with insulin to mean that during the preincubation insulin combines with the diaphragm and that it is only in the bound state that insulin exerts its customary physiological action. In view of the similar effects of preincubation of the tissue with the alkali-metal ions, it is possible that the real significance of these preincubation effects lies in a binding of the agents to the cells. Undoubtedly there is some interaction between insulin or the cations and certain constituents of the cell surface. These results may indicate that insulin or the cations bring about some sustained change in the cell surface, thus making it more permeable to certain solutes such as glucose, and not that insulin or the cations exert their effects only in a bound state. During the preincubation the binding necessary for subsequent action during the final incubation is achieved.

The order in which the alkali metals affect the uptake of glucose by the diaphragm (Table 3) is the same as the order of these ions in the so-called lyotropic or Hofmeister series. It is generally agreed that the series has its origin in the intensity of the electrostatic field around the ions, the small

ions of the same valency having a more intense field than the large ones. In biological reactions, in which the importance of water cannot be overemphasized, it seems that the property of the ions which is the immediate cause of the series arises from a difference in hydration of the ions as a result of the difference of the intensity of the field due to their different sizes. Thus with the univalent ions in question here, the Li^+ ion has the smallest radius, the most intense field, and maximum ionic hydration, whereas the Cs^+ ion has the largest radius, the weakest field and the smallest hydration, if any. It is tempting to correlate the uptake of glucose in the presence of equivalent amounts of the above ions with the amount of water that the ions possibly carry to some active sites on the cell surface, thus altering in a regular way the permeability of those sites to glucose and to other similar solutes.

In conclusion it seems that two separate and distinct processes (one, insulin-independent and the other, insulin-dependent) are possibly involved in the uptake of glucose by the isolated diaphragm of the rat. The process independent of insulin, although markedly influenced by ionic environments, can nevertheless function in the absence of metal ions in the medium. It can also function both under aerobic and under anaerobic conditions. The process dependent on insulin, however, seems to operate best only under aerobic conditions and in the presence of certain metal ions in the incubation medium. There are certain apparent similarities between the action of insulin and of the alkali-metal ions in regulating the glucose metabolism of the diaphragm. It seems, however, that the ways in which they exert their effects are different, two different sites of the cell surface being possibly involved in the two processes. Insulin may act by affecting certain magnesium-dependent sites on the surface, whereas the ions act on certain other sites.

SUMMARY

1. Insulin increases the uptake of glucose by the isolated rat diaphragm only in the presence of certain metal ions in the added medium. It appears that simultaneous participation of Mg^{2+} ions and an alkali-metal ion such as Na^+ is required for the maximum effect of insulin.

2. Mg^{2+} ions seem to be essential for maintaining the tissue sensitive to insulin. Magnesium possibly exerts this effect by preserving the integrity of some components of the cell surface by controlling the activity of some enzyme in the system. Calcium antagonizes magnesium in this respect.

3. The alkali-metal ions have a striking regulatory effect on the basal uptake of glucose by the diaphragm of the rat. In the presence of equivalent amounts of the ions the uptake is highest with Li^+ ions and follows the order

$$Li^+ > Na^+ > K^+ = Rb^+ > Cs^+.$$

In the presence of appropriate amounts of Li^+ ions the uptake is almost as high as that due to insulin from Gey's buffer. The ions exert their regulatory effect even when the tissue has permanently lost its ability to respond to insulin.

4. Preincubation of the diaphragm with the alkali-metal ions leads to the uptake of different amounts of glucose when the tissue is subsequently incubated in Gey's medium, and the uptake follows the order mentioned above.

5. In the presence of equivalent amounts of the alkali-metal ions, the xylose-space of the diaphragm followed the order $Li^+ > Na^+ > K^+$. This suggests that the regulation of the utilization of glucose by the alkali-metal ions is possibly brought about by a regulation of the rate of entry of the sugar from the extracellular fluid to the interior of the cell.

6. It is suggested that two separate and distinct processes (one, insulin-independent and the other, insulin-dependent) are possibly involved in the utilization of glucose by the rat diaphragm.

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The Structure of Cephalosporin C

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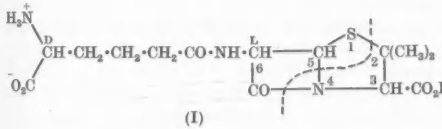
The isolation of cephalosporin C was described by Newton & Abraham (1956). Degradative studies by Abraham & Newton (1956*a*) indicated that cephalosporin C was structurally related to cephalosporin N[(D-4-amino-4-carboxy-*n*-butyl)penicillin; I] (Abraham, Newton & Hale, 1954; Newton & Abraham, 1954; Abraham & Newton, 1954). This paper presents chemical evidence for a definitive structure for cephalosporin C.



(I)

The molecular formula proposed for cephalosporin C ($C_{16}H_{21}O_8N_3S$) had two carbon atoms and two oxygen atoms more than the formula of cephalosporin N (Newton & Abraham, 1956). Cephalosporin C behaved as a monoaminodicarboxylic acid. It contained a residue of D-α-aminoadipic acid, linked to the rest of the molecule through its δ-carboxyl group, and gave 1 mole of carbon dioxide on hydrolysis with hot acid. It yielded L-alanine, valine and glycine, as well as D-α-aminoadipic acid, when hydrolysis was preceded by vigorous hydrogenolysis with Raney nickel. The infrared spectrum of its sodium salt showed a strong band at 5·62 μ, similar to that shown by the penicillins and attributed in the latter to the C=O stretching vibration of the lactam group in the fused β-lactam–thiazolidine ring system (Fig. 1). In these properties cephalosporin C resembled cephalosporin N. Cephalosporin C showed a high degree of resistance to inactivation by penicillinase, but it proved to be a competitive inhibitor of penicillinase (Abraham & Newton, 1956*b*) and an inducer of the synthesis of the enzyme by *Bacillus cereus* and *B. subtilis* (Pollock, 1957).

The close similarities of cephalosporin C and cephalosporin N were accompanied by several striking differences. Cephalosporin C yielded no penicillamine (D-β-mercaptovaline) on hydrolysis and gave 2 moles of ammonia when hydrolysed under conditions in which only 1 mole of ammonia was liberated from cephalosporin N or benzylpenicillin. The valine obtained after treatment of cephalosporin C with Raney nickel was racemic, whereas the valine-yielding fragment of cephalosporin N had the D-configuration. The ultraviolet-absorption spectrum of cephalosporin C sodium salt showed a band with $\lambda_{max.}$ at 260 mμ (log ε 3·95).

We first considered the hypothesis that cephalosporin C contained the skeleton of the characteristic ring system of the penicillins. The infrared-absorption spectrum of cephalosporin C and the ability of the substance to act as a competitive

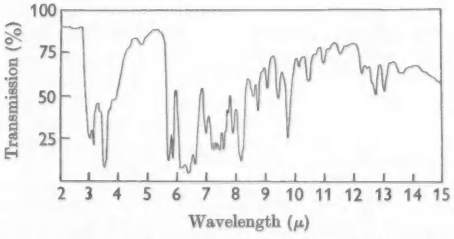


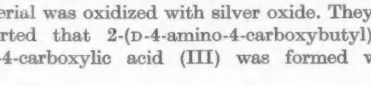
Fig. 1. Infrared-absorption spectrum of cephalosporin C sodium salt (Nujol paste).

inhibitor and an inducer of penicillinase were consistent with this suggestion. It seemed possible to account for the absence of penicillamine from hydrolysates, and for the fact that the valine obtained on hydrogenolysis was racemic, by placing an oxygen function at C-3 in the skeleton of I. A structure of this type also appeared to account for the formation from cephalosporin C, in very small yield, of a substance that resembled penicillaminic acid (β-sulphovaline) in its behaviour on paper electrophoresis at pH 7. This substance was detected after the antibiotic had been subjected to catalytic hydrogenation, the product hydrolysed, and the hydrolysate oxidized with bromine water (Abraham & Newton, 1956*a*). In addition, the structure accommodated the finding that 2 moles of ammonia were liberated on hydrolysis, and that α-oxo*iso*valeric acid

$$[(CH_3)_2CH\cdot CO\cdot CO_2H]$$

was obtained when the antibiotic was treated with Raney nickel at room temperature and the product treated briefly with hot dilute acid (Abraham & Newton, 1958). However, the hypothesis that a modified β-lactam–thiazolidine ring system was present in cephalosporin C threw no light on the nature of the chromophore responsible for the absorption maximum at 260 mμ. The results of more extensive experiments, made possible by the availability of larger amounts of the antibiotic, showed that this hypothesis would have to be abandoned.

Jeffery, Abraham & Newton (1960) reported that δ-amino-δ-carboxyvalerylglycine (II) was formed in small yield when cephalosporin C was hydrolysed with acid and the neutral fraction of the resulting material was oxidized with silver oxide. They also reported that 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (III) was formed when



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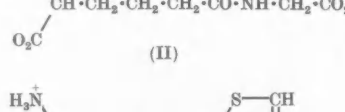
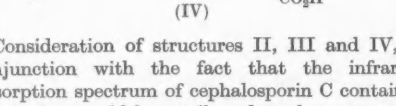


(III)

cephalosporin C was kept in neutral aqueous solution at 37°. δ-Amino-δ-carboxyvalerylglycine had previously been isolated as a degradation product of cephalosporin N, its formation in this case involving hydrolytic opening of the β-lactam ring, decarboxylation of the resulting potential β-aldehydo acid and oxidation of the potential aldehyde to a carboxylic acid (Newton & Abraham, 1954; Abraham & Newton, 1954). The thiazole (III) contained a sequence of carbon, nitrogen and sulphur atoms identical with that in the fragment of cephalosporin N that appears to the left of the broken line dissecting (I). Its formation was understandable if a similar structural fragment occurred in cephalosporin C and if an elimination reaction resulted in fission of the bond between the sulphur atom of this fragment and the remaining portion of the molecule.

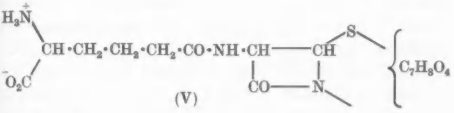
Further evidence for the sequence of atoms near to the side chain of cephalosporin C came from a study of the ninhydrin-positive compounds formed when the antibiotic was treated with Raney nickel at room temperature and the product hydrolysed with N-hydrochloric acid at 100° for 4 min. The resulting mixture was resolved by electrophoresis on paper at pH 7·0 followed by chromatography on paper in butanol–acetic acid–water (4:1:4, by vol.). An acidic component of the mixture corresponded in mobility and R_F with α-aminoadipic acid. Three neutral substances, which gave minor purple spots when coloured with ninhydrin, corresponded with glycine, alanine and valine. A fourth neutral substance gave a major purple spot with R_F 0·054. The latter substance, after elution from the paper, was hydrolysed by further treatment with acid. The hydrolysate contained an acidic compound that behaved like α-aminoadipic acid and a base (R_F 0·09) that gave a grey–brown spot with ninhydrin and migrated towards the cathode at pH 7·0 about 0·8 times as fast as an ornithine marker. The base was indistinguishable, in mobility, R_F and the ninhydrin colour it gave on paper, from an authentic sample of $\alpha\beta$-diaminopropionic acid. It was thus concluded that the substance with R_F 0·054 was the diaminodicarboxylic acid (IV).



(IV)

Consideration of structures II, III and IV, in conjunction with the fact that the infrared-absorption spectrum of cephalosporin C contained bands that could be attributed to the presence of a fused β-lactam ring (5·62 μ) and a monosubstituted amide (6·03 and 6·53 μ), enabled the antibiotic to be assigned, with some confidence, the partial structure (V). This structure contained the skeletons of all three degradation products and could be assumed to give rise to these products by processes that were readily acceptable.

Acceptance of the partial structure (V) left a fragment $C_7H_8O_4$, containing one acidic group, to be accounted for. The formation of DL-valine and of α-oxo*iso*valeric acid on hydrogenolysis of cephalosporin C indicated that five of the seven carbon atoms were associated with the skeleton (VI) or (VII), or possibly a skeleton from which (VII) could be formed by migration of a double bond.



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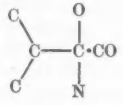

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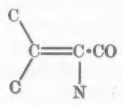


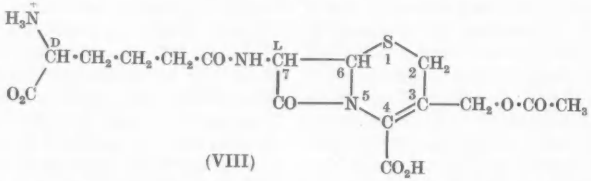
(VII)

The nature of the remaining C_2 fragment was established when 1 mole of acetic acid was shown to be liberated from cephalosporin C by hydrolysis with 1·25 N-sulphuric acid for 1 hr. at 100°, or with N-sodium hydroxide for 1·5 hr. at room temperature. The acetic acid was characterized by the preparation of its *p*-bromophenacyl derivative and by conversion into the corresponding hydroxamic acid and chromatography of the latter on paper. The presence of a band at 5·77 μ in the infrared spectrum of cephalosporin C suggested that the acetic acid was derived from an acetoxyl group. A band at 9·7 μ could then be assigned to the O–C stretching vibration of the CH_3·CO–O–C grouping.

Cephalosporin C gave approximately one *C*-methyl in the Kuhn–Roth determination, and this was accounted for if an acetoxyl group were present in the molecule. The presence, in addition, of a *gem*-dimethyl group [corresponding to the $(CH_3)_2C$ group of the valine and α-oxo*iso*valeric acid obtained as degradation products] did not appear to be excluded by this finding, since the $(CH_3)_2C$ group may give only a fraction of one equivalent of acetic acid in the oxidative procedure (Kuhn & Roth, 1933). No clear evidence for or against the presence of a $(CH_3)_2C$ group was provided by the infrared spectrum of cephalosporin C sodium salt in Nujol. However, on the assumption that a *gem*-dimethyl group was present, no plausible structure that was consistent with the ultraviolet-absorption spectrum of cephalosporin C could be derived from (VI); and no further development of (VII) could be made, since ozonolysis of cephalosporin C under the conditions recommended by Kuhn & Roth (1932) for the estimation of *iso*propylidene groups indicated that a $(CH_3)_2C$:C group was absent. Further, attempts to demonstrate the formation of α-oxo-β-thio*iso*valeric acid on acid hydrolysis of cephalosporin C were unsuccessful. Two crystalline sulphur-containing enolic compounds, which contained no *C*-methyl or nitrogen (subsequently referred to as compounds 1 and 2) were isolated from acid hydrolysates. These compounds appeared to be formed from the C_5 fragment of cephalosporin C that could also give rise to valine, but acceptable structures for them could not be derived from the assumption that the antibiotic C contained sulphur in a five-membered ring.

The question whether a *gem*-dimethyl group was present in cephalosporin C was answered decisively by a nuclear-magnetic-resonance spectrum, for which we were indebted to Mr P. Higham and Dr R. E. Richards. The spectra of the sodium salts of cephalosporin C and benzylpenicillin were determined in aqueous solution, the solvent water being used as an internal standard and the values corrected to the τ scale, on which silicon tetramethyl represents a shift of 10·0 p.p.m. In the spectrum of benzylpenicillin it was possible to account for all the hydrogen present and a large peak at 7·9 p.p.m. was due to the *gem*-dimethyl group. The spectrum of cephalosporin C showed no such peak. A peak of the intensity required for one methyl was present at 7·4 p.p.m., superimposed on a hump due to the combined resonance of the CH_2 and CH groups of the 4-amino-4-carboxy-*n*-butyl side chain. The peak due to this single methyl was in the position expected for the methyl of a CH_3·CO·O group. Broad resonance at 5·9 p.p.m. could be attributed to isolated CH_2 in or on the ring system, although it was not possible to decide whether one or two such groups were present. There was also an indication of resonance at 4·3 p.p.m., corresponding to the CH–CH resonance in benzylpenicillin.

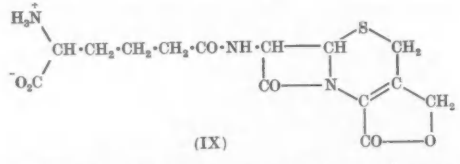
At this stage it occurred to us that the simplest expression of the known properties of cephalosporin C was provided by the structure (VIII), containing a six-membered dihydrothiazine ring. One attraction of this, or a closely related, structure was its ability to account for the formation of a new compound with antibacterial activity, named cephalosporin C_c, when cephalosporin C was kept in 0·1 N-hydrochloric acid at room temperature. Cephalosporin C_c showed no net charge when subjected to electrophoresis on paper at pH 5 or pH 7 and Abraham & Newton (1958) had suggested that it was a lactone. Hydrolysis of the acetyl group in (VIII) would lead to a compound from which an αβ-unsaturated-γ-lactone might readily be formed. A second attraction of (VIII) was that it enabled enolic lactone structures (discussed below) to be postulated for compounds 1 and 2.



(VIII)

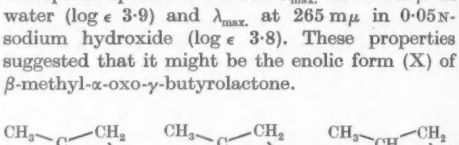
When the structure (VIII) was first considered the direct evidence for it was limited and certain alternatives, in particular a structure with a double bond between C-2 and C-3, were not excluded. But the adoption of (VIII) as a working hypothesis, to be tested by further chemical degradations, soon proved to be fruitful. Before we had made any substantial advance in the subsequent work, Dr Dorothy Hodgkin and Dr E. N. Maslen, who were carrying out an X-ray-crystallographic analysis of cephalosporin C sodium salt, began to discern a sulphur-containing six-membered ring in the molecule. From then on both chemical and X-ray-crystallographical studies made rapid progress. The chemical studies are described below and the X-ray-crystallographical ones by Hodgkin & Maslen (1961).

Cephalosporin C_c was observed to crystallize readily from dilute acetic acid after it had been freed from contaminating acidic products by passage through a column of Dowex 1 (acetate form). Crystalline cephalosporin C_c contained acetic acid of crystallization and the results of elementary analysis indicated that its formula was $C_{14}H_{17}O_6N_3S,CH_3{\cdot}CO_2H$. It gave only one *C*-methyl in the Kuhn–Roth determination (attributable to acetic acid of crystallization) and hence did not contain the acetoxyl group present in cephalosporin C. It showed an ultraviolet-absorption spectrum similar to that of cephalosporin C, but with $\lambda_{max.}$ at 257 mμ. Its infrared-absorption spectrum showed strong bands at 5·62 and 5·69 μ. It was ninhydrin-positive and yielded α-aminoadipic acid on hydrolysis. It contained an ionizable group with $pK_a < 2{\cdot}4$ and a group with pK_a 9·6. These properties were consistent with the view that cephalosporin C_c was the $\alpha\beta$-unsaturated lactone (IX).



(IX)

Treatment of cephalosporin C or cephalosporin C_c with Raney nickel at room temperature, hydrolysis of the resulting material with acid and extraction of the hydrolysate with ethyl acetate yielded a new crystalline degradation product which was named compound 4. Elementary analysis of compound 4 indicated that a possible molecular formula was $C_5H_8O_3$. The compound contained an acidic group with pK_a 7·7 in water at 20°. It gave a cherry-red colour with ferric chloride in aqueous solution and its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 232 mμ in water (log ϵ 3·9) and $\lambda_{max.}$ at 265 mμ in 0·05 N-sodium hydroxide (log ϵ 3·8). These properties suggested that it might be the enolic form (X) of β-methyl-α-oxo-γ-butyrolactone.



(X) (XI) (XII)

β-Methyl-α-oxo-γ-butyrolactone was then synthesized by the method of Fleck, Rossi, Hinder & Schinz (1950). The synthetic product, which Fleck *et al.* (1950) had shown to exist in the enolic form, was indistinguishable from compound 4.

A precursor of β-methyl-α-oxo-γ-butyrolactone, named compound 5, was formed when cephalosporin C_c was treated with Raney nickel. Compound 5 was extracted from the aqueous solution with chloroform. It sublimed in a high vacuum at 100°. Its ultraviolet-absorption spectrum in water showed $\lambda_{max.}$ at approximately 250 mμ. When the pH of the solution was lowered from 3 to 1 the band with $\lambda_{max.}$ at 250 mμ was replaced by a new band with $\lambda_{max.}$ at 216 mμ and this change was reversed when the pH was raised again to 3 (Fig. 2). Compound 5 thus appeared to contain a group which was protonated in the pH range 1–3. It migrated as a base when subjected to electrophoresis on paper in 10% (v/v) formic acid and gave a weak-blue spot when the paper was sprayed with ninhydrin. When it was kept in N-hydrochloric acid at 100° for 30 min. its ultraviolet-absorption spectrum changed irreversibly to that of (X).

These properties suggested that compound 5 was the lactone (XI) of $\alpha\beta$-dehydro-γ-hydroxyvaline. The amino group of (XI), linked to an ethylenic carbon which is itself linked to a lactone carbonyl

group, would have only weakly basic properties, and the pK of compound 5 (about 2) appeared to represent a dissociation constant of the right order of magnitude. The ultraviolet-absorption spectra of compound 5 in neutral and acid solution was also consistent with structure (XI). 2-Methylprop-1-ene-1-carboxylic acid lactone would be expected to show $\lambda_{max.}$ at about 216 mμ, since 2-*cyclo*hexylprop-1-ene-1-carboxylic acid lactone shows $\lambda_{max.}$ in this region (Paist, Blout, Uhle & Elderfield, 1941). But the presence of a free amino group as a substituent in an ethylenic system may have a striking bathochromic effect, $\Delta\lambda$ being 40–50 mμ in some cases (see, for example, Braude, 1945). This effect is associated with the presence of an unshared pair of electrons on the nitrogen atom and is abolished when the latter is protonated in acid solution.

Support for structure (XI) was provided by further experiments with the solution obtained by treating cephalosporin C_c with Raney nickel at room temperature. This solution showed an absorption plateau at 240–250 mμ. Hydrogenation of the solution with Adams catalyst resulted in a disappearance of the absorption. Subsequent hydrolysis with N-hydrochloric acid at 100° gave material which was shown by electrophoresis on paper at pH 7 to contain acidic, neutral and basic ninhydrin-positive substances. One of the basic substances, which gave a brownish colour with ninhydrin and migrated towards the cathode 0·44 times as fast as an ornithine marker, corresponded in colour and position to the lactone of authentic γ-hydroxyvaline (XII). This substance, eluted from the paper after electrophoresis, also showed the same R_F as the lactone of γ-hydroxyvaline (R_{Val}, 0·97) on a paper chromatogram run with butanol–acetic acid–water. On boiling with aqueous ammonia, it was converted into a neutral substance with the same R_F (R_{Ala}, 1·05) as that of γ-hydroxyvaline itself. The formation of γ-hydroxyvaline lactone under these conditions was to be expected if cephalosporin C_c yielded a compound with the structure (XI), or this compound together with an N-acyl derivative of it, on desulphurization. The γ-hydroxyvaline was presumably a mixture of stereoisomers.

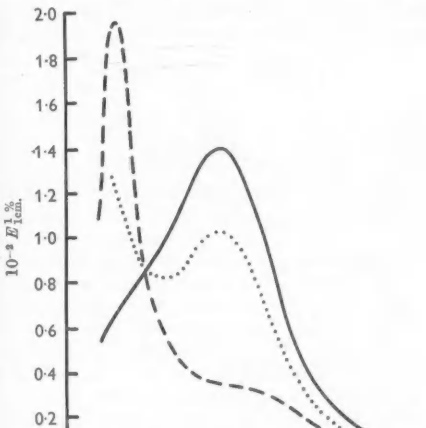


Fig. 2. Ultraviolet-absorption spectrum of compound 5 (not recrystallized). —, In water; ...., in 0·01 N-HCl; – – –, in 0·1 N-HCl.

The identification of the enolic β-methyl-α-oxo-γ-butyrolactone (X) as a degradation product of desulphurized cephalosporin C threw direct light on the nature of compounds 1 and 2 which had been obtained earlier from cephalosporin C itself. When cephalosporin C was heated in 1·25 N-hydrochloric acid at 100° for 1 hr., carbon dioxide was evolved and the absorption band with $\lambda_{max.}$ at 260 mμ disappeared and was replaced by a new band with $\lambda_{max.}$ at 237 mμ. The product responsible for the new absorption band was extracted into ethyl acetate. Although this product crystallized readily from methanol, it was not homogeneous. Chromatography on paper in butanol–acetic acid–water showed that it contained two compounds (1 and 2) with R_F values of 0·81 and 0·85 respectively. When the mixture was subjected to electrophoresis on paper at pH 7·0, compound 1 migrated towards the anode as fast as α-aminoadipic acid and compound 2 migrated more slowly. The two compounds could be revealed on paper by their absorption of ultraviolet light and by the fact that they gave an immediate brown colour when sprayed with aqueous silver nitrate. They were separated on a preparative scale by countercurrent distribution in the system n-butyl acetate–water.

Analytical data showed that a possible formula for compound 1 was $C_{10}H_{10}O_6S$ and that a probable formula for compound 2 was $C_{10}H_{10}O_5S_2$. Neither compound showed any C-methyl in the Kuhn–Roth determination and neither appeared to have optical activity. Compound 1 contained two weakly acidic groups with pK_a values of 6·3 and 9·9 in 50 % (v/v) dimethylformamide. Compound 2 contained two acidic groups with pK_a values of 6·8 and 9·8 in 50 % (v/v) dimethylformamide. The ultraviolet-absorption spectrum of compound 1 showed $\lambda_{max.}$ at 235 mμ in water and $\lambda_{max.}$ at 280 mμ in 0·05 N-sodium hydroxide (Fig. 3). That of compound 2 showed $\lambda_{max.}$ at 243 mμ in water and a broad band between 240 and 330 mμ in 0·05 N-sodium hydroxide (Fig. 4). The infrared spectra of both

compounds showed bands at 3·08 and 5·78 μ which could be attributed to the presence of an OH group and an ester or lactone carbonyl respectively.

Both compounds 1 and 2 gave a strong cherry-red colour with ferric chloride, and gave negative tests with sodium nitroprusside for thiol or disulphide groups. On oxidation with performic acid at $-10°$ they yielded new compounds with R_F values of 0·61 and 0·73 respectively on paper chromatograms run with butanol–acetic acid–water. The new compounds were revealed on paper by their absorption of ultraviolet light and by the fact that they slowly assumed a grey colour when sprayed with aqueous silver nitrate. They were possibly the sulphones of compounds 1 and 2.

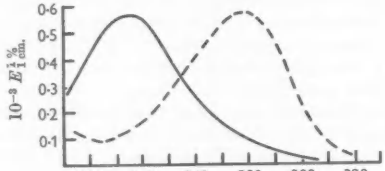


Fig. 3. Ultraviolet-absorption spectrum of compound 1. —, In water; – – –, in 0·05N-NaOH.

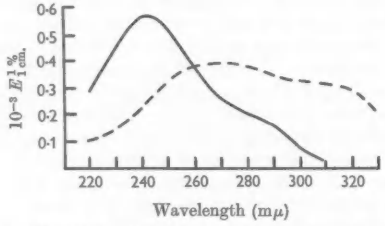
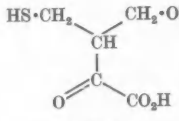


Fig. 4. Ultraviolet-absorption spectrum of compound 2. —, In water; – – –, in 0·05N-NaOH.

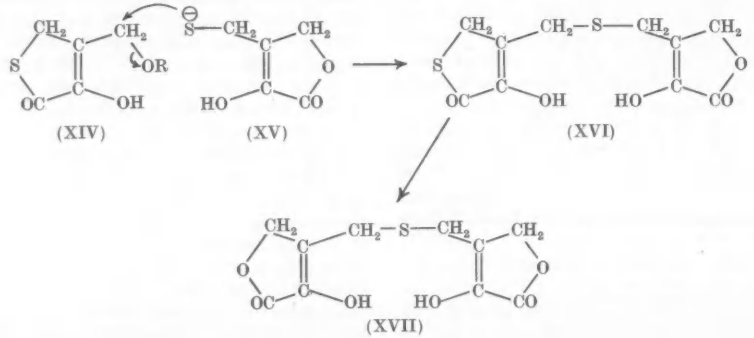
After heating in N-sodium hydroxide at 55° for 10 min. compound 2 gave a strong nitroprusside reaction and a strong smell of hydrogen sulphide was apparent on acidification. After similar treatment compound 1 gave a weak nitroprusside reaction and there was only a faint smell of hydrogen sulphide on acidification. The two compounds also differed in their behaviour when kept in N-hydrochloric acid at 100° for 30 min. Paper chromatography indicated that compound 1 was largely unchanged by this treatment, but that compound 2 was partly converted into compound 1 and a new uncharacterized substance (compound 3) which showed a higher R_F than compound 2 in butanol–acetic acid–water.

Treatment of compound 1 or compound 2 with Raney nickel yielded a product which was indistinguishable from β-methyl-α-oxo-γ-butyrolactone (compound 4) with respect to its ultraviolet-absorption spectrum in water and in 0·05N-sodium hydroxide and was not resolved from the latter when chromatographed on paper in butanol–acetic acid–water or when subjected to electrophoresis on paper at pH 9·0.

The properties of compounds 1 and 2 indicated that they were enolic lactones formed by condensation of two molecules of the C_5 fragment of cephalosporin C. Since they contained no *C*-methyl, but yielded β-methyl-α-oxo-γ-butyrolactone (X) on treatment with Raney nickel, both compounds could be assigned a structure in which the CH_3 group of (X) was replaced by a –S–CH_2 group. They would then be derived from a hypothetical intermediate with the structure (XIII), in which R

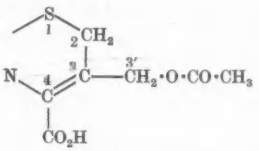


(XIII)



is H or $CH_3 \cdot CO$. The carboxyl group of (XIII) could lactonize with either the thiol group or the potential hydroxyl group to give (XIV) or (XV) respectively. Condensation of (XIV) and (XV), as indicated, would give (XVI), and the latter is suggested as one possible structure for compound 2. $\beta\gamma$-Elimination of sulphur from the ketonic form of the thiolactone ring of (XVI), followed by hydrolytic removal of hydrogen sulphide and relactonization, would yield (XVII). The structure (XVII) is tentatively proposed for compound 1.

The hypothetical intermediate (XIII) in the formation of compounds 1 and 2 could be readily derived from the structure (XVIII). With a view to determining whether cephalosporin C itself contained a double bond in the position of that in (XVIII) the sodium salt of the antibiotic was ozonized in aqueous solution. The solution was then stirred with Raney nickel at room temperature and the resulting volatile material distilled in steam. When the distillate was heated with a solution of 2:4-dinitrophenylhydrazine in 2N-hydrochloric acid an orange-coloured osazone was precipitated in small yield. This osazone was identical with the 2:4-dinitrophenylosazone of authentic hydroxyacetone. Hydroxyacetone is not the only compound from which this osazone can be formed by reaction with 2:4-dinitrophenylhydrazine. However, the isolation of the osazone was consistent with the presence of the grouping (XVIII) in cephalosporin C.



(XVIII)

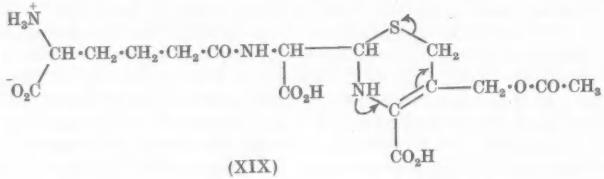
No satisfactory alternative to (XVIII) could be formulated. Thus, a linkage of S to C-3 required that C-2 be joined to C-3′ or C-4 to form a *cyclo*propane ring, since cephalosporin C contained only one *C*-methyl and therefore C-2 could not be a methyl carbon. The resulting structures could account for the formation of valine from cephalosporin C on hydrogenolysis since the *cyclo*propane ring in the amino acid hypoglycin was known to be cleaved by catalytic hydrogenation (Ellington, Hassall & Plimmer, 1958; De Ropp *et al.* 1958; Renner, Jöhl & Stoll, 1958). However, neither of the structures could accommodate a double bond or account for degradation products with the properties of compounds 1 and 2. A linkage of S to C-3′, which also required the presence of a *cyclo*propane ring, failed to account for the retention of sulphur in compounds 1 and 2 since it contained a hemimercaptal grouping. A linkage of S to C-4 was not feasible on either chemical or stereochemical grounds. A modification of (XVIII) in which C-2 and C-3′ were linked to give a *cyclo*propane ring was inconsistent with the presence of a peak due to an isolated CH_2 group in the nuclear-magnetic-resonance spectrum of cephalosporin C. A variant of (XVIII) in which the double bond was between C-2 and C-3 did not account for the isolation of hydroxyacetone 2:4-dinitrophenylosazone from cephalosporin C after ozonolysis and treatment with Raney nickel.

Since cephalosporin C contained only three nitrogen atoms, the nitrogen of (XVIII) was also the β-lactam nitrogen of (V). Combination of (XVIII) and (V) thus led to (VIII). We concluded that the chemical data gave this structure for cephalosporin C a high degree of probability.

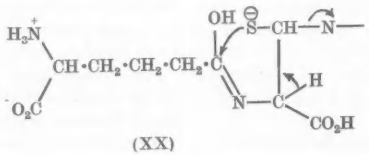
Structure (VIII) accounts for the presence of two strongly acidic groups in cephalosporin C, for the formation of cephalosporin C_c (IX) from cephalosporin C in acid solution at room temperature, for the loss of carbon dioxide in hot acid solution, and for the oxidative degradation of the molecule to δ-amino-δ-carboxyvalerylglycine (II). Reductive removal of the sulphur and the acetoxyl group from (VIII) yields an intermediate from which α-oxo*iso*valeric acid is formed, together with ammonia, on hydrolysis. When hydrogenolysis is accompanied by hydrogenation of a double bond the final product is DL-valine. Hydrolysis of the β-lactam ring of (VIII) leads to (XIX), from the C_5 fragment of which sulphur may be eliminated, as indicated, to form an intermediate (XX) that is converted into the thiazole (III).

Cephalosporin C and cephalosporin C_c show absorption maxima at longer wavelengths (260 and 257 mμ respectively) than would be expected with a normal *N*-acyl αβ-unsaturated amino acid. α-Acetamido-ββ-dimethylacrylic acid was prepared by the method of Abraham, Baker, Chain & Robinson (1949) and showed $\lambda_{max.}$ at 223 mμ. However, in a normal amide the unshared pair of electrons on the nitrogen atom is involved in resonance, whereas in a fused β-lactam ring system there are stereochemical reasons for believing that resonance is suppressed (Woodward, 1949). It may be that suppression of resonance in the β-lactam of cephalosporin C allows the nitrogen of this lactam to exert a considerable bathochromic effect.

When cephalosporin C or cephalosporin C_c was dissolved in 12N-hydrochloric acid at 20° the absorption at 260–257 mμ fell to about 30% of its original value in 8 min. With cephalosporin C_c the resulting solution showed a strong absorption maximum at 216 mμ and a weak broad maximum in the region of 265 mμ. The maximum at 216 mμ disappeared and that at 265 mμ increased when the



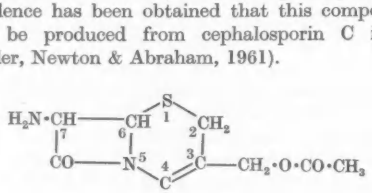
(XIX)



(XX)

pH of the solution was adjusted to 5. Opening of the β-lactam ring in 12N-hydrochloric acid and protonation of the resulting amine nitrogen might be responsible for these changes in absorption.

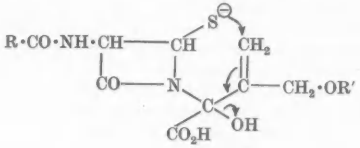
The nucleus of structure (VIII) for cephalosporin C differs from the nucleus of the penicillins in containing a fused β-lactam–dihydrothiazine ring system in place of a fused β-lactam–thiazolidine ring system. The dihydrothiazine ring is a heterocycle that does not appear to have been encountered previously in Nature. The nucleus of the penicillin molecule has been named 6-aminopenicillanic acid (Sheehan, Henery-Logan & Johnson, 1953). We propose the trivial name 7-aminocephalosporanic acid for the nucleus (XXI) of cephalosporin C. Evidence has been obtained that this compound can be produced from cephalosporin C itself (Loder, Newton & Abraham, 1961).



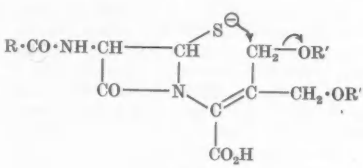
(XXI)

The finding of Arnstein, Morris & Toms (1959) that δ-(α-aminoadipoyl)cysteinylvaline, or the corresponding disulphide, is present in the mycelium of *Penicillium chrysogenum* has raised the possibility that cephalosporin N, or a closely related substance, occupies a special position in the general scheme of penicillin biosynthesis. δ-(α-Aminoadipoyl)cysteinylvaline has a formal resemblance to glutathione and may be synthesized in an analogous manner, by the coupling of α-aminoadipic acid and cysteine, followed by the coupling of the resulting dipepetide with valine. Oxidative cyclization of the tripeptide in the manner postulated by Arnstein & Cawhall (1957), or Birch & Smith (1958), could yield cephalosporin N. Arnstein & Morris (1960) have suggested that benzylpenicillin is formed in *P. chrysogenum* by the action of a transferase on cephalosporin N and that this transferase is lacking in the *Cephalosporium* sp. But it is also possible that the accumulation of cephalosporin N is associated with the fact that the α-aminoadipic acid residue in its side chain has the D-configuration. The *Cephalosporium* sp., but not *P. chrysogenum*, may possess, for example, a racemase by which L-α-aminoadipic acid is converted into the D-isomer at some stage of the biosynthetic process. Arnstein & Morris (1960) isolated α-aminoadipic acid from the mycelium of *P. chrysogenum* and showed that it was mainly the L-enantiomorph.

The similarity in structural pattern of cephalosporin C and cephalosporin N leaves little doubt that the two substances are biogenetically related. The optical configuration at C-7 in cephalosporin C is the same as that at C-6 in the penicillins. Further, X-ray-crystallographic analysis has shown that the hydrogen atoms on the β-lactam ring of cephalosporin C, like those on the β-lactam ring of the penicillins, are *cis* (Hodgkin & Maslen, 1961). It is possible that δ-(α-aminoadipoyl)cysteinylvaline is a common precursor of cephalosporin C, cephalosporin N and benzylpenicillin, and that the biosynthesis of cephalosporin C depends on a mechanism for the oxidation of the *gem*-dimethyl group of the valine residue in the tripeptide. Alternatively, valine may be oxidized before incorporation into the peptide, or an analogue of valine at the appropriate level of oxidation may be formed by a pathway in which valine itself is not an intermediate. Whatever the sequence of events at this stage, structures such as (XXII) or (XXIII) could be envisaged as precursors of the final ring system.



(XXII)



(XXIII)

It is of interest that γ-hydroxyvaline is present in *Kalanchoe daigremontiana* (Pollard, Sondheimer & Steward, 1958) and that a residue of γδ-dihydroxyleucine occurs in the toxic polypeptide phalloidin (Wieland & Schöpf, 1959).

EXPERIMENTAL

Methods

Paper chromatograms were run on Whatman no. 1 paper in the following solvent systems: (*A*) butan-1-ol–acetic acid–water (4:1:4, by vol.); (*B*) butan-1-ol–ethanol–water (5:1:4, by vol.); (*C*) 2-methylbutan-2-ol–propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88) (65:5:30, by vol.) (Isherwood & Cruickshank, 1954); (*D*) butan-1-ol saturated with aq. NH_3 soln. [3 ml. of aq. NH_3 soln. (sp.gr. 0·88) diluted with water to 100 ml.] (Cavallini, Frontali & Toschi, 1949); (*E*) butan-1-ol saturated with 0·1 N-HCl; (*F*) butan-1-ol–ethanol–water (4:1:5, by vol.); (*G*) pentan-1-ol–formic acid–water (3:1:3, by vol.); (*H*) pyridine–ethanol–NH_3 (Herting, Ames & Harris, 1958); (*I*) butan-1-ol–acetic acid–water (4:1:5, by vol.).

Paper electrophoresis on Whatman no. 1 paper (14 v/cm., for 2·5 hr. unless stated otherwise) was carried out as described by Newton & Abraham (1954). The buffers used were: collidine acetate (0·05 M to acetate), pH 7·0; pyridine acetate (0·05 M to acetate), pH 4·5 or 5·0; 10% (v/v) acetic acid, pH 2·2; 10% (v/v) formic acid, pH 1·5; 0·05 M-$(NH_4)_2CO_3$, pH 9·0.

In many cases substances were revealed, after chromatography or electrophoresis on paper, by placing the paper between the viewer and a source of ultraviolet light (230–400 mμ; Corning 9863 filter). Substances which showed strong absorption in the range 240–300 mμ appeared as dark spots.

Electrometric titrations were carried out at 20° in the manner described by Newton & Abraham (1953). Raney nickel was prepared by the method of Pavlic & Adkins (1946) and stored under ethanol at 0°. The amounts of Raney nickel used in different experiments are given as ml. of ethanol-wet product. Antibacterial activities were determined by the hole-plate method, as reported by Abraham & Newton (1956*a*) with *Staphylococcus aureus* or *Salmonella typhi* as the test organism, and cephalosporin C sodium salt as a standard. Analyses were by Weiler and Strauss.

Preparation and paper chromatography of hydroxamic acids. A methanolic solution of hydroxylamine (1·25 N) was prepared by the method of Wingerd (1953), except that the pH was adjusted until the solution gave a green–blue colour on testing with thymol blue.

Samples of aqueous solutions of the acids to be studied (containing 1–5 mg. of each acid) were made alkaline with aq. NH_3 soln. (sp.gr. 0·88) and evaporated to dryness. The salts thus obtained were converted into the corresponding methyl esters by the method of Bergmann & Segal (1956). To the solution of the methyl esters in ether–methanol (about 2 ml.) was added 0·2 ml. of freshly prepared 1·25 N-hydroxylamine. The mixture was kept at 20° for 1 hr. If necessary, 0·1 N-triethylamine in methanol was added drop by drop from time to time until the solution gave a green–blue colour on testing with thymol blue. After 1 hr., 2 μl. of a solution of thymol blue were added to the mixture and the green–blue colour was discharged with CO_2. The solution was then evaporated to dryness in a stream of air and the solid was extracted three times with 50 μl. of dry methanol. The methanolic extracts were evaporated to dryness and the resulting hydroxamic acids dissolved in 50–250 μl. of methanol. Samples (5 μl.) of these solutions were used for paper chromatography. Paper chromatograms of the hydroxamic acids were run in systems *G*, *H* and *I* and the spots were revealed by spraying the papers with 1·0% (w/v) $FeCl_3$ in 95% (v/v) ethanol containing 0·1% of HCl. Formohydroxamic acid showed $R_{\text{acetohydroxamic acid}}$ values in systems *G*, *H* and *I* of 0·73, 0·60 and 0·86 respectively.

Derivatives of cephalosporin C

Cephalosporin C. The antibiotic was used as the crystalline hydrated sodium salt with an activity of 8–10 u./mg. (Newton & Abraham, 1956) (Found: *C*-Me, 3·8; 3·2; acetyl, 10·3. $C_{16}H_{20}O_8N_3SNa,2H_2O$ requires for 1 *C*-Me, 3·2; 1 acetyl 9·0%). A strong band at 8·10 μ in the infrared spectrum of the sodium salt (Fig. 1) could be due to the C–O stretching vibration of an acetoxy group (cf. Jones & Herling, 1956).

Cephalosporin C free acid (Newton & Abraham, 1956) is subsequently referred to as cephalosporin C.

Performic acid oxidation product of cephalosporin C. A solution of performic acid was prepared by adding 30% (w/v) H_2O_2 (0·5 ml.) to AnalaR '98/100%' formic acid (9·5 ml.) and keeping the mixture at 20° for 2 hr. The solution was stored at −10°. Cephalosporin C (80 mg.) was dissolved in formic acid (2 ml.), methanol (0·4 ml.) was added, and the solution cooled to −6°. Cold performic acid solution (6·4 ml.) was then added and the mixture kept at −6° for 2·5 hr. After the addition of 100 ml. of cold water the solution was freeze-dried. The residue was dissolved in water (7 ml.) and the pH of the solution adjusted to 6·4 by addition of 0·1 N-NaOH. The solution was then freeze-dried to give a crude sodium salt (65 mg.). The latter was dissolved in water (0·25 ml.), ethanol (0·25 ml.) was added, and the solution added to a column of Nuchar C (V. A. Howe and Co. Ltd.) (3 cm. × 0·6 cm. diam.). Elution with 50% (v/v) ethanol removed most of the required product in 1 ml. of eluate. Ethanol was added to this eluate until the

mixture remained permanently turbid. The turbidity was removed by centrifuging and ethanol was added drop by drop to the clear supernatant until a further turbidity was seen. The product then separated as a white crystalline sodium salt, which was filtered off and dried in air (28 mg.) (Found: C, 38·1; H, 4·6; N, 8·1; S, 6·5. $C_{16}H_{20}O_9N_2S,2{\cdot}5H_2O$ requires C, 38·5; H, 5·0; N, 8·4; S, 6·4%). The air-dried product showed an activity of 1 u./mg. against *Staph. aureus*. It lost weight (7·1%) corresponding to approximately $2H_2O$ on drying *in vacuo* at room temperature. Its ultraviolet absorption spectrum showed $\lambda_{max.}$ 258 mμ, log ϵ 3·95. The R_F (0·12) of the oxidation product on paper chromatograms (system *A*) was less than that of cephalosporin C (0·19). When subjected to electrophoresis on paper at pH 7·0, the oxidation product migrated towards the anode at the same rate as cephalosporin C. When lead acetate in 20% (w/v) NaOH was added to a solution of the oxidation product, the solution slowly developed a dark colour, whereas a solution of cephalosporin C immediately gave a heavy chocolate-coloured precipitate (PbS) under similar conditions.

The oxidation product was formed under conditions similar to those reported by Hirs (1956) to oxidize methionine quantitatively to its sulphone. However, the infrared spectrum of the sodium salt of the oxidation product in Nujol was similar to that of cephalosporin C sodium salt. It showed strong bands at 5·66, 5·78, 8·08 and 9·68 μ, but did not show bands near 7·6 and 9·0 μ that would be expected for a sulphone. The band at 9·68 μ in the spectrum of the oxidation product was more intense, relative to the bands at 5·66 and 5·78 μ, than was the band at 9·7 μ, relative to the bands at 5·62 and 5·77 μ, in the spectrum of cephalosporin C. The oxidation product may be the sulphoxide of cephalosporin C. Sulphoxides show an absorption band in the 9·7 μ region (see Bellamy, 1958). The band in the spectrum of cephalosporin C at 9·7 μ may be attributed to the O–C stretching vibration of the $CH_2{\cdot}CO{\cdot}OC$ grouping.

Cephalosporin C_c. Cephalosporin C sodium salt (10 g.) was dissolved in water (150 ml.), and the pH of the solution was adjusted to 2·5 by addition, with stirring, of Dowex 50X8 (H form). The mixture was filtered and the resin was washed with about 50 ml. of water. To the filtrate was added AnalaR conc. HCl (25 ml.) and water (65 ml.). The flask containing the acid solution was kept in a water bath at 20° for 18 hr. During this time the extinction of the solution at 260 mμ fell to half its original value. The solution (290 ml.) was then added to a column (46 cm. × 3 cm. diam.) of Dowex 1X8 (200–400 mesh; acetate form). After all the solution had entered the column, the latter was washed with water. The effluent was collected in 10 ml. fractions. Neutral material began to emerge from the column in the 18th fraction and by the 45th fraction the extinction of the effluent at 260 mμ had fallen to a low value. The contents of fractions 18–45 were pooled and concentrated to about 30 ml. *in vacuo* in a rotary evaporator. Acetic acid (6 ml.) was added to the concentrate whereupon crystallization of cephalosporin C_c began immediately. After 2 hr. the crystals were filtered, washed with about 3 ml. of ice-cold 3N-acetic acid, and dried *in vacuo* (yield 3·2 g.). For analysis, a sample of the product (100 mg.) was recrystallized by dissolving it in 1·0 ml. of water and adding 0·18 ml. of acetic acid. After 2 hr. the crystals were filtered and washed with a little ice-cold 3N-acetic acid (yield 50 mg.).

Cephalosporin C_c showed $[\alpha]_D^{24}$ +139° in water (*c*, 1·25). It showed an activity of 1 u./mg. against *Staph. aureus* but less than 0·25 u./mg. against *Salm. typhi*. It contained acetic acid of crystallization which was not removed by drying *in vacuo* at 100° (Found: C, 45·9; H, 5·1; N, 9·8; S, 7·5; *C*-Me, 2·2; acetyl, 12·5. $C_{14}H_{17}O_6N_3S,CH_3{\cdot}CO_2H$ requires C, 46·2; H, 5·1; N, 10·1; S, 7·7; 1 *C*-Me, 3·6; 1 acetyl, 10·4%). The ultraviolet-absorption spectrum of the product in water showed $\lambda_{max.}$ 257 mμ, log ϵ 3·90. The infrared-absorption spectrum (in Nujol) showed strong bands at 3·06, 5·62, 5·69, 6·02 and 6·55 μ.

The nuclear-magnetic-resonance spectrum of cephalosporin C_c was kindly determined by Mr P. Higham and Dr R. E. Richards with a spectrometer operating at 30 Mcyc./sec., the solvent water being used as an internal standard and the values corrected to the τ scale. The spectrum showed an extremely sharp peak at 7·4 p.p.m. This could be assigned to the CH_2 group of the acetic acid of crystallization, since it was intensified, but showed no resolution into more than one component, when free acetic acid (0·5 mole/mole of cephalosporin C_c) was added to the solution. Broad resonance at 5·7 p.p.m. could be attributed to an isolated CH_2 group. The peak at 7·4 p.p.m. was superimposed on a hump due to the combined resonance of the CH_2 and CH groups of the side chain.

The pH of a solution of crystalline cephalosporin C_c (4·9 mg.) in 2 ml. of water was 3·5. Electrometric titration with acid indicated that the substance contained an acidic group with $pK_a < 2{\cdot}4$. On titration with alkali a second acid group (pK_a 4·7, due to acetic acid of crystallization) was detected and a basic group with p*K* 9·6. The equivalent, calculated from the span of the group with pK_a 4·7, was 415±15. Back-titration with acid after the solution had been brought to pH 11·5 and then kept for 1 hr. indicated that a new acidic group had been liberated with pK_a approx 3·6. The liberation of an acidic group in alkaline solution was associated with a change in the ultraviolet-absorption spectrum, $\lambda_{max.}$ increasing to 265 mμ and log ϵ at this wavelength falling to 3·65. A further change in the absorption spectrum occurred when a sample of the alkaline hydrolysate was diluted with 50 vol. of 11N-HCl. The ultraviolet absorption maximum at 265 mμ was replaced by a lower plateau (log ϵ 3·54) and a new maximum appeared at 216 mμ (log ϵ 3·90). These changes may be due to opening of the β-lactam ring in alkaline solution and protonation of the liberated NH group of the dihydrothiazine ring in strongly acid solution.

On paper chromatograms, cephalosporin C_c showed R_F 0·1 in system *B* and R_F 0·25 in system *A*. When subjected to electrophoresis on paper at pH 4·5 or 7·0 it behaved as though it had no net charge. At pH 2·2 or 1·5 it migrated towards the cathode at about half the rate of a leucine marker. Spots were revealed by ninhydrin or observation in ultraviolet light.

In aqueous solution at pH 7 cephalosporin C_c was slowly transformed to an acidic ninhydrin-positive substance which migrated towards the anode approximately as fast as cephalosporin C on electrophoresis on paper at pH 7·0. Visual estimation of the intensity of the spots indicated that about half of the cephalosporin C was transformed in 24 hr. under these conditions. The product was indistinguishable in its electrophoretic behaviour from that formed rapidly from cephalosporin C at pH 11·5.

Degradation products of cephalosporin C

αβ-Diaminopropionic acid. Cephalosporin C sodium salt (50 mg.) in water (3·5 ml.) was stirred with Raney nickel (about 0·25 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and washed with about 2 ml. of water. The combined supernatant and washing was extracted four times with 2 ml. of 1% (w/v) 8-hydroxyquinoline in $CHCl_3$ and then three times with 2 ml. of $CHCl_3$. A sample (0·1 ml.) of the aqueous solution was evaporated to dryness *in vacuo*, the residue dissolved in 0·05 ml. of water, and 5 μl. samples of the resulting solution were used for paper chromatography. The main portion of the solution (5 ml.) was acidified with 0·5 ml. of 11 N-HCl and heated at 100°. Samples (0·1 ml.) were removed from the hot mixture, at successive intervals, and evaporated to dryness *in vacuo*. The residues were dissolved in 0·05 ml. of water, and 5 μl. samples used for chromatography and electrophoresis on paper.

Paper chromatography in system *A* showed that the product of hydrogenolysis (before treatment with hot HCl) was a complex mixture. At least seven ninhydrin-positive substances were present, with R_F values between 0·25 and 0·57. After hydrolysis of the product with HCl at 100° for 4 min. the picture was greatly changed. Electrophoresis on paper at pH 7·0 followed by paper chromatography in a second dimension (system *A*) indicated that the mixture contained two major ninhydrin-positive components. One was a neutral substance with R_F 0·054 and the other corresponded in behaviour to α-aminoadipic acid. During hydrolysis with HCl at 100° for 5 hr. the substance with R_F 0·054 gradually disappeared. At the same time the amount of α-aminoadipic acid increased and a new basic substance appeared which gave a grey–brown spot with ninhydrin, showed R_F 0·09, and migrated towards the cathode at pH 7·0 approx. 0·8 times as fast as an ornithine marker (Table 1). The new substance was indistinguishable in its behaviour under these conditions from αβ-diaminopropionic acid.

Liberation of acetic acid. (*a*) On acid hydrolysis. Cephalosporin C sodium salt (250 mg.) was dissolved in 12 ml. of water, 1·8 ml. of 10 N-H_2SO_4 was added, and the solution heated under reflux at 100° for 1·25 hr. The pH of the solution was then adjusted to 2·2 by the addition of 0·3 N-$Ba(OH)_2$. The precipitated $BaSO_4$ was centrifuged down and washed with water, and the combined supernatant and washing was distilled to dryness *in vacuo*. Electrometric titration of the distillate with 0·1 N-NaOH indicated that the latter contained an acid with pK_a 4·8 (0·8 equiv./mole of cephalosporin C). Evaporation of the titrated distillate yielded a crystalline sodium salt. Paper chromatography of the hydroxamic acid obtained from a small sample of this salt (see Methods section) indicated that the latter consisted almost entirely of acetic acid. The main portion of the sodium salt (40 mg.) was dissolved in 0·5 ml. of water, a solution of 100 mg. of *p*-bromophenacyl bromide in 1·0 ml. of ethanol was added, and the mixture was refluxed for 2 hr. After cooling, the crystalline product was filtered off and dried (37 mg.; m.p. 78°). After recrystallization from 1 ml. of aq. 66% (v/v) ethanol it had m.p. 83°. When this product was mixed with authentic *p*-bromophenacyl acetate (m.p. 85°) the m.p. was 84°.

(*b*) On alkaline hydrolysis. Cephalosporin C sodium salt (50 mg.) was dissolved in N-NaOH (1 ml.) and the solution left at 16° for 1 hr. The solution was then added to a column (5 cm. × 1·2 cm. diam.) of Dowex 50 X 8 (200–400 mesh; H form), and the column washed with water (about 5 ml.). The organic acid in the eluate was converted into the corresponding hydroxamic acid. Paper chromatography indicated that the latter consisted of acetohydroxamic acid together with a trace of formohydroxamic acid.

Hydroxamic acids from other products of hydrolysis. Two uncharacterized products of hydrolysis of cephalosporin C were detected on paper chromatograms in the form of the corresponding hydroxamic acids (referred to as *P* and *Q*). Cephalosporin C sodium salt (25 mg.) was hydrolysed with N-H_2SO_4 (1 ml.) at 100° for 1 hr. The pH was adjusted to 2·0 by addition of 0·3 N-$Ba(OH)_2$ and the precipitated $BaSO_4$ removed. Organic acids in the supernatant were converted into the corresponding hydroxamic acids. The products gave four spots on paper chromatograms. Two spots corresponded with aceto- and formo-hydroxamic acids, the former being about three times as intense as the latter. A third spot (*P*) showed $R_{\text{acetohydroxamic acid}}$ values of 0·4, 0·77 and 0·86 respectively in systems *G*, *H* and *I*. The fourth spot (*Q*) showed $R_{\text{acetohydroxamic acid}}$ values of 0·11, 0·37 and 0·76 respectively. The compounds in the hydrolysate of cephalosporin C which were responsible for the formation of *P* and *Q* were not extractable by ether and did not distil in steam.

Isolation of compounds 1 *and* 2. Crude cephalosporin C sodium salt (2·5 g.; about 65% pure) was dissolved in water (35 ml.) and converted into cephalosporin C by adding sufficient Dowex 50 X 8 (H form) to the stirred solution to bring the pH, at equilibrium, to 2·7. About 2·5 g. of damp resin was required. The mixture was filtered and the resin was washed on the filter with water. To the clear filtrate (41 ml.), containing cephalosporin C, were added 2 N-HCl (94 ml.) and water (15 ml.) to give 150 ml. of solution which was 1·25 N to HCl. The acid solution was heated for 1 hr. in a flask on a boiling-water bath in a slow stream of N_2. The CO_2 liberated was absorbed in standard NaOH. Back-titration of the NaOH showed that 0·9 mole of CO_2 was evolved per mole of cephalosporin C. The ultraviolet-absorption spectrum of a sample taken from the solution of cephalosporin C immediately after mixing with the 2 N-HCl showed $\lambda_{max.}$ 260 mμ, $E^{1\%}_{1\,cm.}$ 106. After the solution had been heated for 1 hr. at 100°, it showed $\lambda_{max.}$ 240 mμ and $E^{1\%}_{1\,cm.}$ 108.

The hydrolysate was cooled and extracted twice with 150 ml. of ethyl acetate. Water (100 ml.) was added to the extract and the mixture was evaporated *in vacuo* until the ethyl acetate had distilled and about 98 ml. of water phase remained. Measurements of ultraviolet-absorption spectra showed that virtually all the material in the hydrolysate with $\lambda_{max.}$ at about 240 mμ had been extracted into the ethyl acetate.

The aqueous solution (95 ml.) obtained from the ethyl acetate extracts was transferred to a smaller flask and evaporated to dryness *in vacuo*, leaving a crystalline mass attached to the walls of the flask by a gum. Cold water (7 ml.) was added, which dissolved most of the gum and detached the crystals. The crystals were filtered off and then dissolved in hot methanol (10 ml.). On concentrating the methanol to about 4 ml. and cooling the solution, crystallization began. After 1 hr. the crystals were filtered and washed with a little ice-cold methanol (yield 130 mg.).

The mother liquor was concentrated to about 1 ml. and yielded a second crop of crystals (40 mg.).

The crystalline product (400 mg., prepared from several batches of cephalosporin C) was subjected to counter-current distribution in the solvent system *n*-butyl acetate–water, 14 ml. of the upper phase and 20 ml. of the lower phase being used in each tube of the machine and the upper phase being mobile. At the start of the experiment the product was loaded equally into tubes 0 and 1. After 100 transfers, combined samples of 0·07 ml. of the upper phase and 0·1 ml. of the lower phase from every second tube were diluted to 3 ml. with ethanol and the extinctions of the resulting solutions measured at 237 mμ. The curve obtained by plotting *E* against tube no. showed two bands, almost completely resolved, with maxima at tube 65 (*E* 1·08) and tube 91 (*E* 1·06). The contents of tubes 52–76 were pooled and evaporated to dryness *in vacuo*, leaving crystalline *compound* 1 (138 mg.), which was recrystallized from about 17 ml. of hot methanol (yield 120 mg.). The contents of tubes 80–95 were pooled and evaporated similarly, leaving crystalline *compound* 2 (202 mg.). This product was recrystallized by dissolving it in about 2·5 ml. of warm methanol and adding 9 ml. of hot water to the solution. After the mixture had cooled the crystals were filtered and washed with a little cold water (yield 173 mg.).

Compound 1. This compound (m.p. 148–149°) crystallized from methanol or water in needles which appeared to lose birefringence on drying. The air-dried crystals (from water) lost 7% of their weight on drying at 80° in a high vacuum. Material dried at 80° was used for analysis (Found: C, 46·1; H, 4·1; S, 11·4; no *C*-Me. $C_{10}H_{10}O_6S$ requires C, 46·3; H, 3·9; S, 12·4%). Compound 1 showed no detectable optical activity in ethyl acetate ($[\alpha]_D^{20} < \pm 3°$). Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 235 mμ ($E^{1\%}_{1cm.}$ 580) in water and $\lambda_{max.}$ 280 mμ ($E^{1\%}_{1cm.}$ 580) in 0·05N-NaOH. Its infrared-absorption spectrum (in Nujol) showed strong bands at 3·08 μ (OH), 5·76 μ (lactone C=O), 5·90 μ (C=C?), 8·78 μ and 12·88 μ. Its equiv. [by electrometric titration in 50% (v/v) dimethylformamide] was 135±10. An aqueous solution of compound 1 gave no precipitate on the addition of 2:4-dinitrophenylhydrazine in 2N-HCl.

Dimethyl ether of compound 1. When compound 1 (10 mg.) in methanol (0·2 ml.) was treated with a small excess of ethereal diazomethane it was converted into a new product (presumably the dimethyl ether) which crystallized on evaporation of the solvent. After recrystallization from $CHCl_3$–di*iso*propyl ether the new product (6·2 mg.) had m.p. 71–72°. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 230 mμ both in ethanol and in 0·05N-NaOH in 95% (v/v) ethanol. In the latter solvent an absorption band with $\lambda_{max.}$ at 278 mμ appeared after 20 hr. at 20°, presumably due to hydrolysis of an OMe group. The infrared spectrum of the dimethyl ether (in $CHCl_3$) showed strong bands at 5·70 μ (lactone C=O) and 5·95 μ (C=C?).

Desulphurization of compound 1. Raney nickel (about 0·1 ml.) was added to a solution of compound 1 (10 mg.) in water (1 ml.) and 0·1N-NaOH (0·35 ml.). The mixture (pH 7) was stirred for 15 hr. at room temperature. The Raney nickel was removed by centrifuging and the supernatant, after adjustment to pH 4, shaken twice with 1 vol. of ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and the solvent then removed in a stream of N_2. The crystalline residue (3 mg.) was sublimed at 45° and 0·07 mm. The sublimate was indistinguishable from compound 4 (α-oxo-β-methyl-γ-butyrolactone) in m.p. (88°), ultraviolet-absorption spectrum in water and in 0·05N-NaOH, rate of migration towards the anode on paper electrophoresis at pH 9·0 and R_F on paper chromatography in system *A*.

Compound 2. The air-dried compound (m.p. 178–179°) was dried at 80° in a high vacuum (loss of wt. 6·7%) for analysis. (Found: C, 43·4; H, 3·8; S, 23·1; no *C*-Me. $C_{10}H_{10}O_5S_2$ requires C, 43·8; H, 3·7; S, 23·3%). The compound showed no detectable optical activity. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 234 mμ ($E^{1\%}_{1cm.}$ 580) in water and a broad band between 240 and 330 mμ ($E^{1\%}_{1cm.}$ 400 at 265 mμ) in 0·05N-NaOH. Its infrared spectrum (in Nujol) showed strong bands at 3·05 (OH), 5·77 μ (lactone C=O?), 5·90 μ (C=C?), 5·97 μ (thiolactone C=O?), 6·12 μ, 8·75 μ and 9·70 μ. Its equiv. [by electrometric titration in 50% (v/v) dimethylformamide] was 140±10. An aqueous solution of compound 2 gave no precipitate on addition of 2:4-dinitrophenylhydrazine in 2N-HCl.

Desulphurization of compound 2. A neutral aqueous solution of compound 2 (20 mg.) was stirred with Raney nickel under the conditions described for compound 1. A portion (3·5 ml.) of the resulting solution (total vol. 4·5 ml.) was extracted with ethyl acetate and yielded a crystalline product (2·0 mg.) which was indistinguishable from authentic α-oxo-β-methyl-γ-butyrolactone (compound 4). To the remainder of the solution (1 ml.) was added $NaIO_4$ (10 mg.) The mixture was kept at 20° for 2 hr. and freeze-dried, and 2:4-dinitrophenylhydrazine in 2N-HCl was added to the distillate. Orange–yellow 2:4-dinitrophenylhydrazone precipitated (2 mg.), which was partly crystalline. On paper chromatograms run under the conditions described by Lynn, Steele & Staple (1956) this product yielded three yellow spots corresponding in R_F values with the 2:4-dinitrophenylhydrazones of formaldehyde, acetaldehyde and *iso*butyraldehyde. *iso*Butyraldehyde and formaldehyde might have been formed by periodate oxidation of the diol $(CH_3)_2{\cdot}CH{\cdot}CH(OH){\cdot}CH_2{\cdot}OH$.

Oxidation of compounds 1 *and* 2 *with performic acid.* A sample (2 mg.) of compound 1 was dissolved in 0·05 ml. of AnalaR formic acid and 0·01 ml. of methanol, and the solution cooled to −10°. Performic acid solution (0·16 ml., prepared as described earlier) was added and the mixture kept at −10° for 2 hr. Water (1 ml.) was added and the solution freeze-dried. The residue was dissolved in 0·2 ml. of water. Compound 2 was oxidized in a similar manner. Samples (5 μl.) of the resulting solution were used for paper chromatography in system *A*. Detection of spots by their absorption of ultraviolet light showed that compound 1 had R_F 0·77 and compound 2 had R_F 0·84, whereas the R_F values of the corresponding oxidized compounds were 0·66 and 0·73 respectively. When the paper was sprayed with 1% (w/v) $AgNO_3$, compounds 1 and 2 were revealed immediately as light-brown spots, whereas the corresponding oxidized compounds gave grey spots which appeared after about 30 min.

Effect of hydrochloric acid at 100° *on compounds* 1 *and* 2. Samples (0·8 mg.) of compounds 1 and 2 in 1·25N-HCl (0·2 ml.) were heated in sealed tubes at 100° for 1 hr., and 0·5 μl. samples of the resulting solutions (1 and 2 respectively) were analysed by paper chromatography and paper electrophoresis. Solution 1 showed only a single spot in-

distinguishable from that shown by the original compound 1. Solution 2 showed a spot in the position occupied by the original compound 2 (R_F 0·84 in system *A*; migration 4·7 cm. towards the anode at pH 7·0) and two further spots, the first corresponding to compound 1 (R_F 0·77 in system *A*; migration 7·0 cm. towards the anode at pH 7·0) and the second to a new product, compound 3 (migration 2·0 cm. towards the anode at pH 7·0). The $R_{\text{compound 2}}$ values of compounds 1 and 3 in system *E* were 0·8 and 1·1 respectively. Compound 3, like 1 and 2, was revealed on paper by its absorption of ultraviolet light and the fact that it gave a brown colour when sprayed with aq. $AgNO_3$. Compounds 2 and 3 gave a red colour when the paper was sprayed with a 0·1% solution of 2:3:5-triphenyltetrazolium chloride in butan-1-ol saturated with water, dried, and then sprayed with 10N-NaOH–ethanol–butan-1-ol (1:4:5, by vol.). Compound 1 showed no colour under these conditions.

Synthesis of β-mercapto-α-oxoisovaleric acid 2:4-dinitrophenylhydrazone. An attempt to remove the benzyl group from the *S*-benzyl derivative of β-mercapto-α-oxo*iso*valeric acid (Hems, Holland & Robinson, 1949) with Na in liquid NH_3 was unsuccessful. Desulphurization occurred and the product was α-oxo*iso*valeric acid.

β-Bromo-α-oxo*iso*valeric acid (2·5 g.) (Hems *et al.* 1949) in water (8 ml.) was neutralized by the addition of solid $(NH_4)_2CO_3$, and the solution added slowly to a solution of aq. N-NH_3 soln. which had been saturated with H_2S at 0°. The mixture was kept for 15 min. at room temperature, and ethanol (4 vol.) was then added. No product separated from this solution when it was kept overnight (cf. the synthesis of β-mercaptopyruvic acid by Parrod, 1947). The solution was then adjusted to pH 3 with 6N-HCl, evacuated to remove H_2S, and 2:4-dinitrophenylhydrazine in 2N-HCl added until no further hydrazone was precipitated. *β-Mercapto-α-oxoisovaleric acid 2:4-dinitrophenylhydrazone* crystallized in fine plates (m.p. 143–144°, decomp.) from aqueous ethanol (Found: C, 38·3; H, 4·1; S, 8·8. $C_{11}H_{12}O_6N_4S,H_2O$ requires C, 38·2; H, 4·6; S, 9·2%). The product lost weight (5·2%) corresponding to 1 H_2O on drying *in vacuo* at 100°. It dissolved readily in aq. N-NH_3 soln. and the solution became deep red on addition of sodium nitroprusside. A sample (1·6 mg.) was dissolved in 1 ml. of ethanol, 20 ml. of water was added, and the solution hydrogenated for 24 hr. in the presence of 50 mg. of Adams catalyst (L. Light and Co. Ltd.) (cf. Meister & Abendschein, 1956). The catalyst was removed by filtration, the filtrate evaporated to dryness, and the residue dissolved in 0·1 ml. of water. A sample (5 μl.) of the resulting solution gave a strong spot corresponding to valine when chromatographed on paper in system *A*.

The 2:4-dinitrophenylhydrazone of β-mercapto-α-oxo-*iso*valeric acid behaved similarly to that of α-oxo*iso*valeric acid when subjected to paper electrophoresis at pH 7·0 (migration 2·5 cm. towards the anode) or to paper chromatography in system *C* (R_F 0·71), or system *D* (R_F 0·80). When samples (5 μl.) of solutions of these compounds were mixed with 30% (w/v) H_2O_2 (2 μl.) immediately before application to the paper, the 2:4-dinitrophenylhydrazone of α-oxo*iso*valeric acid appeared to be unchanged, whereas that of β-mercapto-α-oxo*iso*valeric acid was partly converted into a new compound (presumably the sulphonic acid) which migrated 5·0 cm. towards the anode at pH 7·0.

2:4-Dinitrophenylhydrazones of products of hydrolysis of cephalosporin C. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl to the solution (7 ml.) obtained by washing the crude mixture of compounds 1 and 2 with water (see above) yielded a heavy orange-coloured amorphous precipitate. The crude 2:4-dinitrophenylhydrazone contained S and most of it was acidic, being soluble in water at pH 7 and reprecipitated on addition of acid (Found: S, 10·25%) (cf. Abraham & Newton, 1958). On catalytic hydrogenation with Adams catalyst, as described above, it yielded a small amount of an amino acid that behaved like valine on paper chromatography in system *A*. However, on paper chromatography in systems *C* and *D* it formed an almost continuous yellow streak from the origin and showed no spot in a position corresponding to the 2:4-dinitrophenylhydrazone of β-mercapto-α-oxo*iso*valeric acid.

Amorphous precipitates were also formed when 2:4-dinitrophenylhydrazone in 2N-HCl was added to alkaline hydrolysates of cephalosporin C (0·2N-NaOH for 4 hr. at 20°), or of compounds 1 and 2 (N-NaOH for 10 min. at 55°) or to an aqueous solution of cephalosporin C (pH 7) which had been kept at 37° for 48 hr. All these precipitates formed streaks on paper chromatography in systems *C* and *D* and attempts to isolate a crystalline product from them were unsuccessful.

Isolation of α-oxoisovaleric acid 2:4-dinitrophenylhydrazone. A solution of cephalosporin C sodium salt (50 mg.) in 3·5 ml. of water was stirred with Raney nickel (about 0·2 ml.) for 20 hr. at 20°. The Raney nickel was filtered and washed with 1·5 ml. of water. To the combined filtrate and washing was added 2·5 ml. of 3N-HCl, and the solution heated in a boiling-water bath for 4 min. Addition of 2:4-dinitrophenylhydrazine in 2N-HCl (about 2 ml.) to the cooled solution resulted in the rapid formation of an orange crystalline precipitate (4 mg.). After crystallization from hot water this product had m.p. 194–196°. The m.p. was not depressed on admixture with authentic α-oxo*iso*-valeric acid 2:4-dinitrophenylhydrazone (m.p. 194–196°). The two products were indistinguishable on paper chromatograms run in system *C* or *D* and when subjected to electrophoresis at pH 7·0. X-ray powder photographs of the two products were kindly taken by Miss Joan Shipley and Dr Dorothy Hodgkin and reported to be identical.

Isolation of compound 4. (*a*) A solution of cephalosporin C sodium salt (200 mg.) in water (14 ml.) was stirred with Raney nickel (about 1 ml.) for 18 hr. The Raney nickel was removed by centrifuging and washed with 5 ml. of water. The ultraviolet-absorption spectrum of the combined supernatant and washing showed a continuous rise in extinction from 290 to 220 mμ. Hydrochloric acid (0·1 vol., 12N) was added and the resulting solution kept at 100° for 20 min. During this treatment the extinction fell at 290–260 mμ and rose to a maximum at 232 mμ. The solution was then shaken twice with 1 vol. of ethyl acetate. The combined extracts were dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue (10 mg.) consisted of a crystalline product contaminated with a brown oil. On sublimation at 60° and 0·07 mm. it yielded a white crystalline sample of compound 4, m.p. 86–90° (5·5 mg.).

(*b*) To a solution of cephalosporin C sodium salt (1 g.) in water (20 ml.) was added 6·35 ml. of 1·04N-HCl. The solution was kept at 20° for 4 days and its pH then adjusted to 5·5 by the addition of 0·2N-NaOH (26 ml.). Raney nickel (about 5 ml.) was added and the mixture stirred for 18 hr. The Raney nickel was removed by centrifuging and washed with 10 ml. of water. The combined supernatant

and washing was shaken twice with 1 vol. of $CHCl_3$. The solvent was evaporated *in vacuo*, leaving a mixture of crystals and thick oil (65 mg.). Sublimation of the mixture at 100° and 0·07 mm. gave a white crystalline product whose ultraviolet-absorption spectrum in water and dilute HCl was that of compound 5 (Fig. 2). To the aqueous phase remaining after the extraction with $CHCl_3$ was added 0·1 vol. of 12N-HCl. The solution was heated at 100° for 20 min., cooled, and shaken twice with 1 vol. of ethyl acetate. The extract was dried over Na_2SO_4 and the solvent removed *in vacuo*. The residue yielded a white crystalline sample (40 mg.) of compound 4, m.p. 88–90°, on sublimation at 60° and 0·07 mm. (Found: C, 53·2; H, 5·6%; equiv. 115±5. $C_5H_6O_3$ requires C, 52·6; H, 5·3%; equiv. 114.).

Compound 4 could be detected on paper in 50 μg. quantities as an ultraviolet-light-absorbing spot which became more intense after the paper had been sprayed with 0·01N-NaOH, or as a brown spot after the paper had been sprayed with ammoniacal $AgNO_3$ and then heated for 3 min. at 100°. On paper chromatograms run in system *A*, it showed R_F 0·88. When subjected to electrophoresis on paper it showed no significant migration at pH 6·8 but migrated towards the anode at pH 9·0. In all these properties it was indistinguishable from β-methyl-α-oxo-γ-butyrolactone (m.p. 87–89°), synthesized by the method of Fleck *et al.* (1950). X-ray powder photographs of the synthetic product and compound 4 were taken by Dr Dorothy Hodgkin and Mr R. Diamond and reported to be identical. Both crystalline products were unstable, undergoing detectable decomposition with the formation of oily material within 2 days (cf. Fleck *et al.* 1950).

Ozonolysis of cephalosporin C. A solution of cephalosporin C sodium salt (20 mg.) in water (3 ml.) was cooled to 0°, and ozonized O_2 (containing about 6% of O_3) passed through the solution. Ozonization was continued for 15 min. after O_3 appeared in the exit gas (test with KI); N-$KMnO_4$ (3 ml.) was then added and the mixture heated under reflux for 7 min. (cf. Kuhn & Roth, 1932). The resulting solution was distilled from a Markham still, and the distillate collected in 2:4-dinitrophenylhydrazine in 2N-HCl. No precipitation of 2:4-dinitrophenylhydrazone occurred. It was concluded that the *iso*propylidene grouping was not present in cephalosporin C.

Ozonolysis followed by desulphurization with Raney nickel. Ozonized O_2 (containing about 6% of O_3) was passed through a solution of cephalosporin C sodium salt (50 mg.) in water (3·5 ml.) at 0° for 1 hr. The solution was then stirred with Raney nickel (about 0·2 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and the supernatant distilled in steam, successive 12 ml. portions of distillate being collected in tubes containing 1 ml. samples of a solution of 2:4-dinitrophenylhydrazine in 2N-HCl. The solutions in tubes 1 and 2 became heavily opalescent and that in tube 3 faintly opalescent. The contents of tubes 1–4 were combined and heated at 100° for 30 min., when a flocculent orange-coloured osazone precipitated. The osazone was filtered, washed with water and dried *in vacuo* (2 mg.). It crystallized from warm pyridine in needles, m.p. 308–310°.

One drop of authentic hydroxyacetone [60% (w/w) solution in methanol from L. Light and Co. Ltd.] was added to 4 ml. of water, the mixture steam-distilled, and a 2:4-dinitrophenylosazone prepared from the distillate in the manner described for the product from cephalosporin C. Authentic hydroxyacetone 2:4-dinitrophenylosazone showed m.p. 307–310° (cf. Bülow & Seidel, 1924). On admixture with the osazone from cephalosporin C the m.p. was 307–310°. X-ray powder photographs of the two products were kindly taken by Dr E. N. Maslen and reported to be identical.

Desulphurization followed by ozonization. Cephalosporin C sodium salt (75 mg.) in water (5 ml.) was stirred with Raney nickel (about 0·5 ml.) for 18 hr. at 20°. The Raney nickel was removed by centrifuging and washed with 1 ml. of water. The combined supernatant and washing was shaken four times with 2 ml. of 1% (w/v) 8-hydroxyquinoline in $CHCl_3$ and then three times with 2 ml. of $CHCl_3$. Ozonized O_2 (6% of O_3) was passed through the clear aqueous solution at 0° for 1 hr. The solution was then distilled in steam and the distillate collected in a solution of 2:4-dinitrophenylhydrazine in 2N-HCl until no further precipitation of 2:4-dinitrophenylhydrazone occurred. The hydrazone was centrifuged down, washed with water and dried (7·2 mg.). It was dissolved in $CHCl_3$ (1 ml.) and added to a column (15 cm. × 1 cm. diam.) packed with a slurry made by mixing 14 g. of Bentonite (Hopkin and Williams Ltd.) and 3·5 g. of kieselguhr as described by Elvidge & Whalley (1955). On elution with $CHCl_3$, part of the crude hydrazone moved down the column as a yellow band. Evaporation of the eluate yielded 4·0 mg. of a crystalline orange–yellow product which had m.p. 158–166° after recrystallization from 95% (v/v) ethanol. On admixture with authentic acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 168°) the m.p. was 162–168°. Subsequent elution of the column with $CHCl_3$ containing 3% (v/v) of ethanol removed a second and light-yellow hydrazone (1·3 mg.). The latter had m.p. 154–157°. On admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone (m.p. 162–164°) the m.p. was 154–158°. The first hydrazone was indistinguishable from acetaldehyde 2:4-dinitrophenylhydrazone and the second from formaldehyde 2:4-dinitrophenylhyrazone when chromatographed in heptane saturated with phenoxyethanol on Whatman no. 7 paper treated with 10% (v/v) phenoxyethanol in acetone as described by Lynn, Steele & Staple (1956).

The failure to detect the formation of acetone on ozonolysis of Raney-nickel-treated cephalosporin C indicated that the treatment with Raney nickel did not yield a product which contained the group $(CH_3)_2C{=}C$. However, treatment with Raney nickel followed by brief hydrolysis with acid resulted in the production of some α-oxo*iso*valeric acid. The latter may therefore have been derived from a group such as $H_2C{:}C(CH_2){\cdot}CH(N){\cdot}CO_2H$, formed from cephalosporin C by hydrogenolysis and migration of a double bond. The small amount of formaldehyde formed when ozonolysis was carried out after hydrogenolysis might have come from a structure of this type. The origin of the small amount of acetaldehyde obtained under these conditions is uncertain. Conceivably it was formed by degradation of an unstable keto acid which remained when a terminal methylene group was removed as formaldehyde.

Degradation products of cephalosporin C_c

Hydrogenolysis with Raney nickel. To 50 mg. of crystalline cephalosporin C_c was added water (3·5 ml.) and Raney nickel (0·36 ml.). The mixture was stirred at 15° for 18 hr.

Most of the Raney nickel was then removed by centrifuging and the supernatant (pH 6·6) was filtered through Whatman no. 42 paper. The precipitated Raney nickel was washed first with water (the clarified washing being added to the filtrate) and then with $CHCl_3$.

Compound 5. The combined filtrate and washing referred to above was extracted three times with 1 vol. of $CHCl_3$, the $CHCl_3$ washing of the Raney nickel being used for the first extract. The extracts were dried over Na_2SO_4 and evaporated to dryness in a stream of N_2, giving feathery crystals of compound 5 (3·6 mg.). The unrecrystallized product showed m.p. 70–75°, $\lambda_{max.}$ 248 mμ, $E^{1\%}_{1cm.}$ 140. The crystalline material was unstable in air but appeared to be stable in a dilute solution in benzene frozen at −20°.

Compound 5 migrated about 0·87 times as fast as isoleucine towards the cathode when subjected to electrophoresis on paper at pH 1·5, but at pH 2·3 it migrated only 0·33 times as fast as isoleucine. Compound 5 could be detected as a dark spot by viewing the papers in ultraviolet light.

Desulphurized cephalosporin C_c. The aqueous solution, which remained after the removal of compound 5 with $CHCl_3$, was extracted three times with 1 vol. of 1% (w/v) oxine in $CHCl_3$, and then freed from oxine by three extractions with 1 vol. of $CHCl_3$. Evaporation of the aqueous layer *in vacuo* left 17 mg. of a crude product (referred to as desulphurized cephalosporin C_c) whose ultraviolet-absorption spectrum showed a marked inflexion at 240 mμ with $E^{1\%}_{1cm.}$ 156. When subjected to electrophoresis on paper at pH 4·5 or 1·5, this product was indistinguishable from cephalosporin C_c. When chromatographed on paper in system *A* it showed a major spot (coloured with ninhydrin) with R_{Ala} 0·92 and a minor spot with R_{Ala} 0·74. One of these spots may have been due to desthiocephalosporin C_c. R_{Ala} of cephalosporin C_c in system *A* was 0·61.

Compound 4 (*1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone*). Desulphurized cephalosporin C_c (16 mg.) was heated with 0·7 ml. of 1·3 N-HCl at 100° for 1 hr. The ultraviolet-absorption spectrum of the hydrolysate now showed $\lambda_{max.}$ at 233 mμ, $E^{1\%}_{1cm.}$ 201. The cooled hydrolysate was extracted three times with 0·5 vol. of ethyl acetate. The extract was dried over $CaCl_2$ and evaporated to dryness in a stream of N_2, giving crystals of compound 4 (4 mg.). This product underwent partial decomposition when kept in a desiccator for several days, but after recrystallization from a mixture of benzene and light petroleum (1:1, v/v) it appeared to be considerably more stable. When the product was subjected to paper chromatography in system *A* and to electrophoresis on paper at pH 9·0, its behaviour was indistinguishable from that of authentic 1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone.

αβ-Diaminopropionic acid. The aqueous residue which remained after extraction of compound 4 from the acid hydrolysate of desulphurized cephalosporin C_c was studied by paper chromatography in system *A*. The chromatogram showed three ninhydrin-positive spots. Spot 1 had R_F 0·054 and spot 2 had the same R_F (0·11) as αβ-diaminopropionic acid. The third spot was not distinguished in R_F from a mixture of alanine and α-aminoadipic acid (Table 1). When the hydrolysate was subjected to electrophoresis on paper at pH 7·0 it was resolved into five ninhydrin-positive spots. One was due to an acidic substance that behaved like α-aminoadipic acid and one to material that showed no net charge. The three remaining spots, which were of minor intensity (spots 2, 4 and 5, Table 1), were due to basic substances. The substance responsible for spot 2 was not resolved from an authentic sample of αβ-diaminopropionic acid and gave the same grey–brown colour as the latter with ninhydrin.

The aqueous residue was hydrolysed further with 6 N-HCl at 110° for 6 hr. and the product again studied by chromatography and electrophoresis on paper. Spot 1 had disappeared whereas spot 2 had become of major intensity and the spot due to α-aminoadipic acid had also increased in intensity. Spots 4 and 5 had undergone little change. Desulphurized cephalosporin C_c, unlike the material obtained by treatment of cephalosporin C with Raney nickel, yielded no detectable amount of valine on hydrolysis.

γ-Hydroxyvaline. Spot 4 (referred to above) did not appear when the hydrolysate was freed from HCl by evaporation *in vacuo* and the residue boiled with aq. 2 N-NH_3 soln. for 5 min. before being subjected to electrophoresis on paper. This property suggested that it might be due to γ-hydroxyvaline lactone. However, since substantial amounts of compounds 4 and 5 had been found in the hydrolysate, only a relatively small yield of γ-hydroxyvaline lactone could be expected (see structures X, XI and XII). A further experiment was therefore carried out in which desulphurized cephalosporin C_c was hydrogenated with Adams catalyst before the hydrolysis with HCl.

Cephalosporin C_c (50 mg.) in 4·0 ml. of water was treated with Raney nickel at 20° as described above. Compound 5 was extracted with $CHCl_3$ and the aqueous residue was freed from nickel by treatment with oxine as described previously. A small sample was taken from the solution for the measurement of its extinction at 250 mμ. Adams catalyst (50 mg.) was added to the remainder of solution (3·8 ml.), and the mixture was hydrogenated at atmospheric pressure. After 20 hr. the solution was freed from

Table 1. *Behaviour of degradation products of cephalosporin C and cephalosporin C_c on paper chromatography and electrophoresis*

Electrophoresis was for 1·5 hr. at 14 v/cm. Details of experimental conditions are given in the Methods section. Spots referred to in the Table are described in the Experimental section. Roman numerals refer to structures given in the text.

Product	R_F in system *A*	Mobility* (cm.) at pH 7 towards	
		Anode	Cathode
(IV) (spot 1)	0·054	—	0·7
αβ-Diaminopropionic acid† (spot 2)	0·11	—	6·5
γ-Hydroxyvaline lactone† (XII) (spot 4)	0·46	—	3·4
γ-Hydroxyvaline	0·30	—	0·7
Alanine	0·31	—	0·7
α-Aminoadipic acid	0·33	5·5	—
Valine	0·50	—	0·7
Spot 5	—	—	11·5
Ornithine marker	0·11	—	7·3
Histidine marker	—	—	2·7

* Glucose was carried 0·7 cm. towards the cathode by endosmosis under the conditions used.

† Grey–brown colour with ninhydrin.

the catalyst. The extinction of a sample of this solution at 250 mμ indicated that reduction of the chromophore originally present in desulphurized cephalosporin C_c was about half complete. The solution was then hydrogenated for a further 20 hr. with fresh Adams catalyst (50 mg.). Measurements of ultraviolet-absorption spectra then indicated that the chromophore was virtually destroyed, and that no compound 4 was formed on heating the solution with N-HCl at 100° for 30 min. The solution was evaporated to dryness, the residue was hydrolysed in N-HCl at 110° for 16 hr., and the HCl removed *in vacuo*. The product of hydrolysis was dissolved in 0·5 ml. of water. A sample (8 μl.) of the hydrolysate was subjected to electrophoresis on paper at pH 7·0 and the paper sprayed with ninhydrin. The hydrolysate gave four major spots, due to an acidic substance (α-aminoadipic acid), neutral material and two basic substances. The bases corresponded with spots 4 and 5 (Table 1). A trace of αβ-diaminopropionic acid (spot 2) was also detected.

A small amount of the product responsible for spot 4 was isolated by preparative paper electrophoresis. Fifteen 4 μl. samples of the hydrolysate were spotted on to the paper at 1 cm. intervals. After electrophoresis, three guide strips, spaced equally along the paper, were cut out and sprayed with ninhydrin to locate spot 4. The material in this position was eluted from the remainder of the paper with water. The eluate was evaporated to dryness and the residue dissolved in 24 μl. of water. One half of this solution was heated in a sealed tube with 4 μl. of aq. NH_3 soln. (sp.gr. 0·88) at 100° for 15 min., the contents of the tube were evaporated to dryness, and the residue was dissolved in 12 μl. of water. Samples (4 μl.) of the untreated and NH_3-treated solutions were subjected to electrophoresis on paper at pH 7·0 and paper chromatography in system *A*. The product in the untreated sample was indistinguishable in behaviour from authentic γ-hydroxyvaline lactone (Table 1). The product in the treated sample was indistinguishable from γ-hydroxyvaline.

The basic substance responsible for spot 5 has not been characterized. Conceivably it is 2-hydroxymethyl-*n*-propylamine [$CH_3 \cdot CH(CH_2 \cdot OH) \cdot CH_2 \cdot NH_2$]. The *iso*butylamide of phenylacetyl-L-alanine is one of the products of hydrogenolysis of benzylpenicillin (Kaczka & Folkers, 1949).

SUMMARY

1. Cephalosporin C ($C_{16}H_{21}O_8N_3S$) has been degraded by hydrolysis, hydrogenolysis and oxidation.

2. Degradation products obtained from one half of the molecule include carbon dioxide, δ-amino-δ-carboxyvalerylglycine, 2-(D-4′-amino-4′-carboxy-*n*-butyl)thiazole-4-carboxylic acid and αβ-diaminopropionic acid.

3. Degradation products obtained from the second half of the molecule include acetic acid, γ-hydroxyvaline, α-oxo*iso*valeric acid and 1-hydroxy-2-methylprop-1-ene-1-carboxylic acid lactone.

4. Consideration of the degradation products, together with the ultraviolet- and infrared-absorption spectra, nuclear-magnetic-resonance spectrum and behaviour on electrometric titration of cephalosporin C, has led to a definitive structure for the antibiotic. This structure contains a fused β-lactam–dihydrothiazine ring system in place of the β-lactam–thiazolidine ring system of the penicillins.

5. Cephalosporin C_c, formed from cephalosporin C in 0·1 N-hydrochloric acid at room temperature, is deacetylcephalosporin C lactone.

6. The biogenesis of cephalosporin C is considered in relation to that of cephalosporin N and other penicillins.

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We should like to record our thanks to those who informed us, during the course of this work, of their interesting speculations about the structure of cephalosporin C. None of the structures suggested to us contained the dihydrothiazine ring though in one, suggested by Dr W. A. Sexton, the acetoxyl group of cephalosporin C was placed on the γ-carbon atom of a valine-yielding fragment.

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The X-ray Analysis of the Structure of Cephalosporin C

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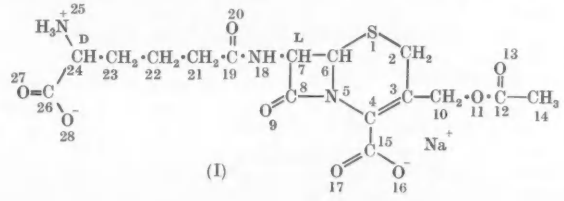
Crystallographic measurements on the sodium salt of cephalosporin C were first made in order to determine the molecular weight of this compound soon after it was isolated by Newton & Abraham (1955). The crystals were then too small for detailed work, but the measurements showed that the lattice had one very short unit-cell dimension of 4·9 Å, a feature that suggested that it might be possible to solve the crystal structure by direct methods, without the introduction of atoms heavier than those already present. As more material was obtained and larger crystals grown, additional unsuspected complications in the crystal lattice were observed and these have hindered the precise solution of the structure. The positions now found for the atoms in the crystal give good general agreement with the observed X-ray intensities but are not defined with great accuracy;



(I)

they correspond with the structural formula (I) described by Abraham & Newton (1961) in the preceding paper.

Preliminary crystallographic examination

Crystalline samples of the ammonium, barium, calcium, potassium and sodium salts of cephalosporin C were prepared by Abraham & Newton; those of the sodium salt were most promising, the ammonium salt rather less so, the rest microcrystalline. Experiments on growing good crystals were only successful for the sodium salt. The method which gave the best results was as follows:

Samples ranging from 1 to 5 mg. of the salt were dissolved in one drop more than the minimum of water. Ethanol was then added in excess. In the intermediate concentrations crystals grew slowly over a period of some weeks and well-ordered crystals up to $1{\cdot}5 \times 0{\cdot}25 \times 0{\cdot}1$ mm.^{3} were obtained. They were easily removed after replacement of the mother liquor with absolute ethanol or after suction filtration.

Even the best crystals were of low reflecting power and deteriorated noticeably in the X-ray beam; no reflexions were observed beyond a spacing limit of 1·04 Å. Many crystals, particularly those which had grown rapidly, showed serious distortions; some of the earlier recrystallized specimens gave reflexions over 1 cm. long on Weissenberg photographs.

The crystals were laths on (001), elongated along the b axis. The crystal data are as follows:

$$a = 38{\cdot}88 \pm 0{\cdot}15 \text{ Å}, \quad b = 4{\cdot}99 \pm 0{\cdot}04 \text{ Å},$$

$$c = 25{\cdot}65 \pm 0{\cdot}10 \text{ Å}, \quad \beta = 115° 25 \pm 15',$$

space group, C2, for which Z is 4. From the earlier measured density, $D_m = 1{\cdot}38$, the molecular weight of the asymmetric unit is calculated to be twice the figure 467 ± 15, which corresponds with the formula preferred on analysis, $C_{16}H_{22}O_8N_3SNa,2H_2O$ (calculated molecular weight 475).

In the intensity distribution it was observed that $h0l$ reflexions were very weak and $h1l$ reflexions rather weak, for l odd, whereas for higher values of k there was no marked distinction between l-odd and l-even reflexions. The effect suggested that the asymmetric unit consisted of two molecules separated by a translation of $\sim c/2$ but having rather different y parameters.

The structure analysis is based on 1428 X-ray reflexions recorded on multiple film Weissenberg photographs and measured by eye.

Structure analysis

Both the course of the structure analysis and the accuracy achieved were much affected by the limited character of the available data. In spite of the short b cell dimension it proved impossible to solve the structure in projection alone and three-dimensional calculations were undertaken. Fig. 1 shows in outline the stages of the analysis.

Three evaluations of the three-dimensional Patterson distribution were made with terms modified by different sharpening functions. All three illustrate the character of the lattice suggested by the intensity distribution; the whole pattern repeats to a first approximation in the interval $c/2$, the pseudo-origin being more drawn out in the b direction than the true origin.

Of the three distributions the first, I, is very diffuse and was of little use; the modification

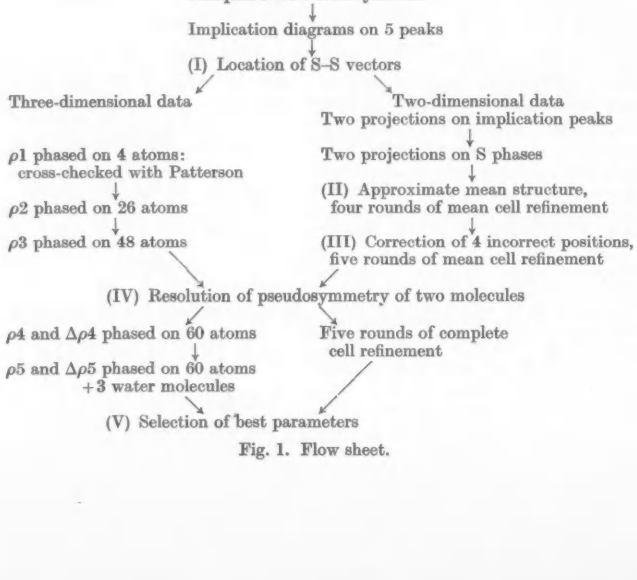

Sharpened Patterson synthesis
Implication diagrams on 5 peaks
(I) Location of S–S vectors
Three-dimensional data
Two-dimensional data
Two projections on implication peaks
ρ1 phased on 4 atoms:
cross-checked with Patterson
Two projections on S phases
ρ2 phased on 26 atoms
(II) Approximate mean structure,
four rounds of mean cell refinement
ρ3 phased on 48 atoms
(III) Correction of 4 incorrect positions,
five rounds of mean cell refinement
(IV) Resolution of pseudosymmetry of two molecules
ρ4 and Δρ4 phased on 60 atoms
Five rounds of complete
cell refinement
ρ5 and Δρ5 phased on 60 atoms
+3 water molecules
(V) Selection of best parameters

Fig. 1. Flow sheet.

function here reduced the terms approximately to those expected from atoms at rest. The third, III, and sharpest version, corresponding with point atoms at rest, was calculated by Dr S. Abrahamson after the main structure analysis here described was complete. The second, II, the distribution actually used, is nearer to III in character; the modification function chosen here, $100 \sin^4 \theta \exp(-9{\cdot}0 \sin^2 \theta)$, was similar to that used in the analysis of serine (Shoemaker, Barieau, Donohue & Lu, 1953) with some adjustment of the exponential function to reduce diffraction ripples. Here the peaks are well resolved; corresponding peaks in the two halves of the Patterson distribution are alternatively high and sharp in one half, and low but extended in the other, thus making it possible to determine where the co-ordinates of atoms in the two molecules differ appreciably. The section in this function at $y = 0$ is shown in Fig. 2.

Of the prominent peaks in the section, $y = 0$, the six highest (numbered 1–6 in Fig. 2) were selected for trial as possibly representing S–S vectors. Implication diagrams were plotted to show the coincidence of the peak pattern surrounding each of these with peaks surrounding the origin, only peaks above 100 on the arbitrary Patterson scale being included. Seventy-three good coincidences throughout the distribution were observed for 3, 55 for 1 (later identified as an O–O vector), whereas relatively few were found for the other peaks. Further, the peak pattern surrounding 3 (Fig. 3) clearly mapped a six-membered ring. This could be correlated directly with the suggestion made in the same period by E. P. Abraham & G. G. F. Newton (personal communication) that a six-membered ring was present in cephalosporin C. It is perhaps worth observing that peak 3 is actually the highest peak in the later, sharper Patterson distribution III, which would have identified it directly as the S–S vector.

It was clear that many of the other atomic positions in the crystal were defined by the S-light atom vectors isolated in the implication diagram shown in Fig. 3, but their selection as a whole was not immediately obvious in the complex situation thus revealed. Also, whereas the implication diagram defined the x and z co-ordinates, it left the sign of the y co-ordinates of the corresponding peaks indeterminate. Accordingly, initial trial calculations were based on the projection data only.

The first approximate electron-density projection was based on signs given by 53 of the more likely implication peaks selected as possible atomic sites. A difference map suggested the removal of eight of these and the addition of ten others. Further structure-factor calculations showed very poor agreement for l-odd reflexions and these were omitted in the next few rounds of calculation, which led accordingly to a mean structure corresponding with a one-molecule asymmetric unit. Parallel with the series based on the peaks in the implication map, an approximate projection was calculated by using signs calculated on the proposed sulphur atom positions only. In this series only the 55 large terms which were likely to have the same phase as the sulphur contributions were included. These were terms for which the unitary structure factor exceeded 0·1, and the sulphur contribution exceeded 0·5 of its maximum possible value. One large reflexion, $20\bar{1}0$, had a sulphur contribution 0·4 maximum and a separate map was evaluated including this term.

Together, the electron-density projections and implication maps considerably limited the possible distribution of the atoms in the crystal. It became clear that the six-membered ring carried substituents on four of the atoms and that two of these, 7 and 8, could be extended to another possible position forming a β-lactam ring. Beyond them a chain could be traced having the form of the adipic acid side chain known to be present in the antibiotic. A second chain, at position 3, was more obscure, but of length equivalent to acetoxymethyl. On the fourth position, a group of peaks suggested a carboxyl group a short distance from a Na^+ ion. The first orientation derived for this group was soon found to be incorrect by the calculation of a series of further refinements of the projected electron-density and difference maps. But in outline the structure derived at this point has been confirmed by subsequent three-dimensional calculations and the parallel chemical investigation.

The refinement of the electron-density projection at this stage is illustrated by Fig. 4*a–c*. Fig. 4*a* shows a difference map calculated for the structure as first outlined, Fig. 4*b*, a difference map calculated leaving out the uncertain atoms connected with the carboxyl group, Fig. 4*c* the subsequent map derived by using $h0l$ terms with l even only. Fig. 4*a* and *b* give some of the evidence which led to the reorientation of the carboxyl group and movement of the Na^+ ion from a site essentially that occupied by one atom of the carboxyl group to that first thought to be occupied by a water molecule. Fig. 4*c* defines the molecule as a whole to a first approximation, together with the Na^+ ion and possible sites for water molecules. R, the agreement factor, at this stage was 26·6 % on observed reflexions only.

Parallel with the calculation of the electron density in projection, calculations of the electron density in three dimensions were made. Here the complete unit-cell data had to be employed throughout. The first stage was independent of the projection evidence. Positions were derived from the implication diagram for four atoms, the

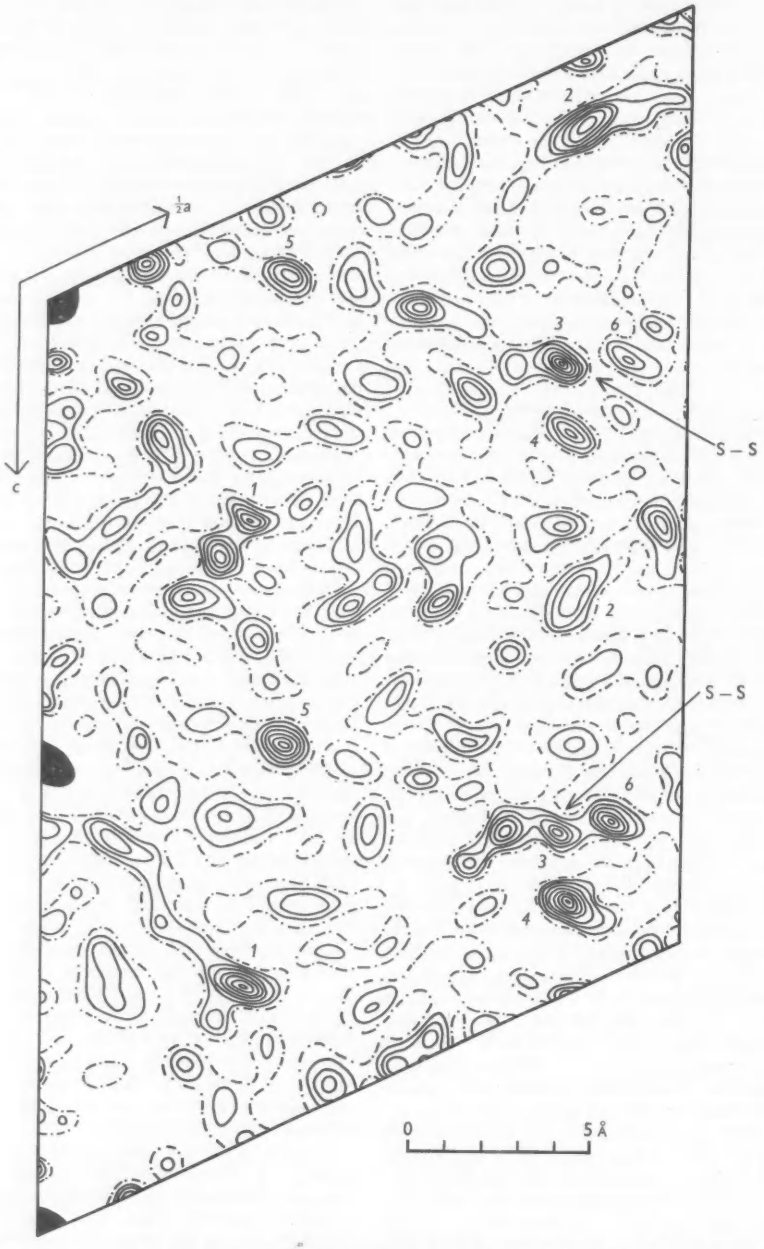


Fig. 2. Section at $y = 0$ in the three-dimensional Patterson distribution II. S–S indicates a peak corresponding to the sulphur–sulphur vector.

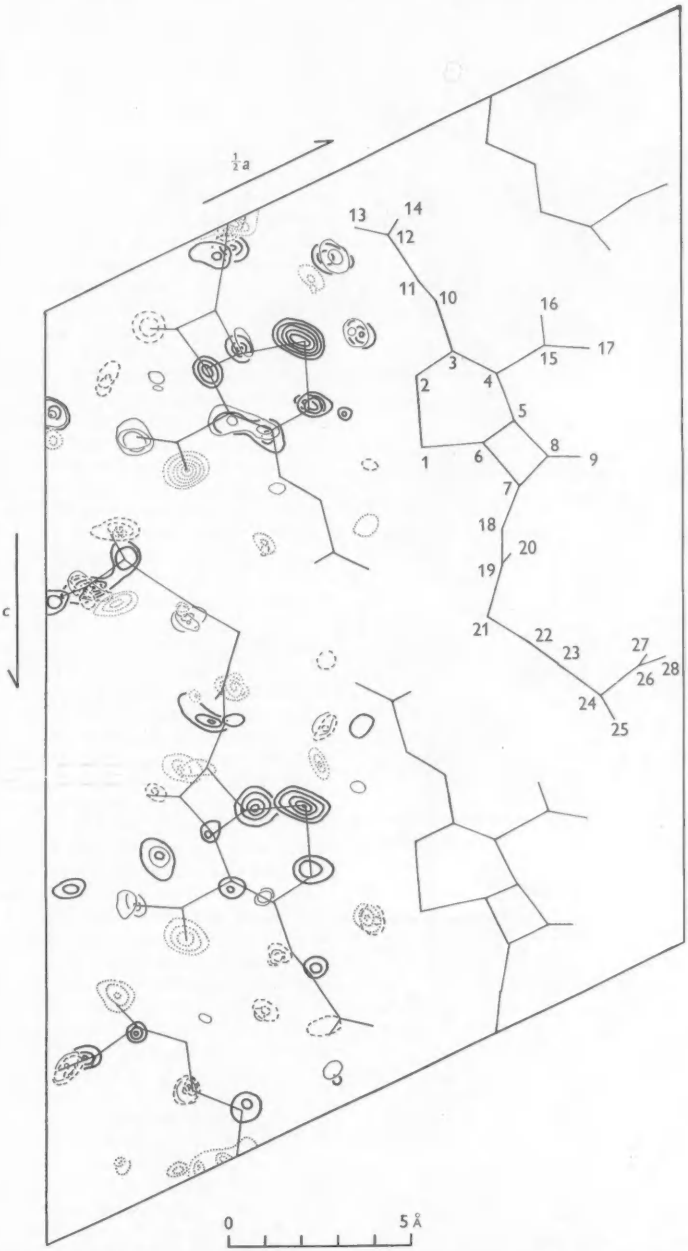


Fig. 3. Implication diagram on peak 3 of Fig. 2. The numbering in this figure corresponds with the numbering of the atoms in Table 1.

Fig. 4. (*a*) Difference synthesis, (010), in fourth round of the projection refinement. The four atoms thought to be incorrectly placed are ringed. Contour interval, 0·5 e/Å^3. (*b*) Difference synthesis, (010), on eighth round of the projection refinement showing the effect of omitting doubtful atoms. Contour interval, 1 e/Å^3. (*c*) Electron-density projection on (010) obtained through the mean cell refinement. Contour interval, 1 e/Å^2. The broken contour is at 2 e/Å^2, below which the contours are omitted. In these figures the correctly oriented molecule is drawn superimposed on the contours. + indicates position chosen for water molecules and the sodium ion.

sulphur atoms in the two independent molecules, *a* and *b*, and two others thought then to be sodium ions, actually oxygen atoms of carboxyl groups. Phase angles were calculated on these positions, the two oxygen atoms being weighted as sodium atoms.

From the following electron-density distribution 26 atomic positions were selected and used in the next phasing calculations and the series was continued, as shown in Fig. 1, by the progressive selection of 48, 60 and 63 atomic positions. The selection was never automatic. In the first synthesis there were peaks present corresponding to every atom in the molecule, ranging from $1\frac{1}{4}$ to $3\frac{1}{2}$ e/Å^3 in height, but these were accompanied by a set of peaks related to them by a false plane of symmetry through $y = 0$, and by an almost equal number of completely spurious peaks up to 2 e/Å^3 in height. The selection of only 26 atomic sites from among these was perhaps over-cautious; examination of the series shows that in 38 cases the present chosen position was higher than the mirror-image peak and in only 18 cases was the false image substantially higher. The completely spurious peaks did not, in general, have false symmetry peaks associated with them, and a direct cross-check with the Patterson synthesis enabled most of them to be eliminated.

The later rounds of calculation proceeded fairly smoothly with small complications. In the 26 atom series only two of the mirror-image peaks were higher than the true peaks; in the 48 atom series, only one was, C-6 (*a*), which had accidentally been inserted at the wrong mirror-image site. Although then represented by a low peak, it did not show up as well as was expected at the correct site. The main problem in the last two stages proved to be the selection of the water-molecule positions. Until these were inserted differently in the two half-cells the atomic positions belonging to the two crystallographically non-identical molecules tended to drift in opposite directions, making different bond-lengths long and short. This tendency was reduced once different water molecule situations were proposed. At the 60 atom stage all the atoms in both molecules, the sodium ions and one water molecule similarly placed in the two half-cells were inserted. Near the (*b*) molecule there were two peaks in the expected position for the water molecules of $2\frac{1}{2}$ and 2 e/Å^3. Near the (*a*) molecule there were three peaks of 1·8, 1·7 and 1·6 e/Å at *X*, *Y* and *Z* in Fig. 5; these are too near to be simultaneously occupied. In the 63 atom distribution positions *X* and *Y* were weighted as if occupied by $\frac{1}{2}$ molecule of water. A small peak of 1 e/Å^3 still persisted at *Z*.

Similar effects were observed in the later refinement stages of the electron-density projection on (010) which includes all *h*0*l* reflexions, not only

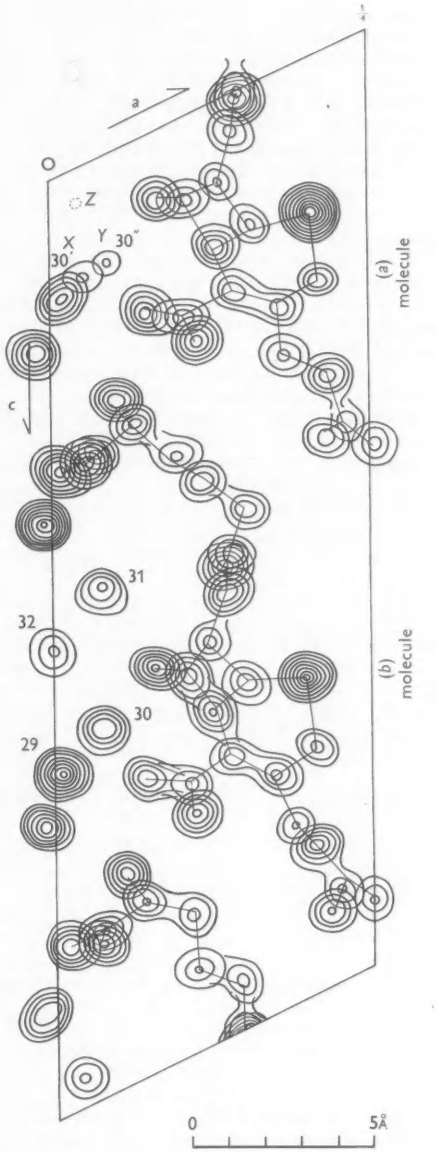


Fig. 5. The arrangement of the molecules in the crystal of sodium cephalosporin C projected on the *b* plane. Sections in the electron-density distribution, $\rho 5$, are shown projected over the atomic positions. *X*, *Y* and *Z* are possible water molecule positions. Contour interval, 1 e/Å^3. The numbers in this figure correspond with the numbers of atoms in Table 1.

water molecules and the sodium ion.

those with l even. While the water molecules near the (b) molecule of cephalosporin C appeared to occupy definite positions those near the (a) molecule were represented by a blurred peak covering both positions X and Y of the three-dimensional distribution, together with a small peak at Z. Also, the atoms near the doubtful water molecule belonging to the (a) molecule appeared in negative regions in difference syntheses with positive peaks on either side, as if they too could occupy alternative positions.

It seems clear that there is some disorder in the arrangement of the water molecules between the (a) type molecules. In any one asymmetric unit either the sites Z and X or Y but not all three may be occupied by water molecules and the nearest atoms of cephalosporin C seem to be displaced a little to correspond. This disorder may be responsible for the weakly reflecting character of the crystals which limits the X-ray data. It, in turn, is presumably due to the packing problems posed by the awkward molecular shape.

The progress of the structure analysis as a whole is illustrated by Fig. 6 which shows the general improvement of the reliability index, R, in the different types of structure-factor calculation carried out. On the three-dimensional data, the

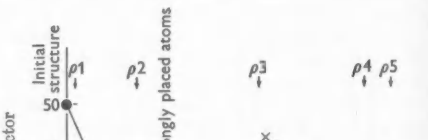


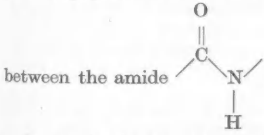
Fig. 6. Reliability index during the refinement of sodium cephalosporin C. ●, $h0l$, l even; ○, $h0l$, l complete; ×, hkl.

Table 1. *Atomic co-ordinates*

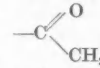
	Atom		x/a	y/b	z/c
1	S	(a)	0·2055	0·988	0·1681
		(b)	0·1990	0·926	0·6595
2	C	(a)	0·2100	0·942	0·2442
		(b)	0·2056	0·891	0·2379
3	C	(a)	0·1788	0·821	0·2480
		(b)	0·1744	0·760	0·7477
4	C	(a)	0·1438	0·823	0·2116
		(b)	0·1403	0·780	0·7012
5	N	(a)	0·1290	0·952	0·1552
		(b)	0·1276	0·888	0·6422
6	C	(a)	0·1582	0·116	0·1529
		(b)	0·1525	0·084	0·6323
7	C	(a)	0·1337	0·110	0·0882
		(b)	0·1226	0·026	0·5705
8	C	(a)	0·1063	0·885	0·0884
		(b)	0·1048	0·800	0·5919
9	O	(a)	0·0840	0·717	0·0736
		(b)	0·0805	0·582	0·5719
10	C	(a)	0·1818	0·671	0·3020
		(b)	0·1891	0·651	0·8092
11	O	(a)	0·2182	0·875	0·3475
		(b)	0·2029	0·886	0·8385
12	C	(a)	0·2303	0·813	0·4057
		(b)	0·2281	0·884	0·9048
13	C or O	(a)	0·2547	0·017	0·4482
		(b)	0·2523	0·123	0·9269
14	O or C	(a)	0·2122	0·602	0·4094
		(b)	0·2179	0·666	0·9188
15	C	(a)	0·1055	0·714	0·2127
		(b)	0·1083	0·538	0·7076
16	$O^{\frac{1}{2}-}$	(a)	0·1155	0·495	0·2447
		(b)	0·1103	0·428	0·7449
17	$O^{\frac{1}{2}-}$	(a)	0·0735	0·817	0·1863
		(b)	0·0719	0·726	0·6812
18	N	(a)	0·1436	0·019	0·0394
		(b)	0·1368	0·927	0·5266
19	C	(a)	0·1484	0·216	0·0030
		(b)	0·1411	0·129	0·4932
20	O	(a)	0·1476	0·498	0·0096
		(b)	0·1338	0·340	0·4999
21	C	(a)	0·1469	0·134	0·9497
		(b)	0·1530	0·967	0·4486
22	C	(a)	0·1127	0·087	0·9100
		(b)	0·1188	0·875	0·3945
23	C	(a)	1·1094	0·992	0·8500
		(b)	0·0959	0·145	0·3591
24	C	(a)	0·0731	0·995	0·8130
		(b)	0·0632	0·071	0·3016
25	N	(a)	0·0578	0·226	0·7770
		(b)	0·0486	0·304	0·2678
26	C	(a)	0·0403	0·883	0·8212
		(b)	0·0341	0·962	0·3151
27	$O^{\frac{1}{2}-}$	(a)	0·0371	0·643	0·8303
		(b)	0·0255	0·721	0·3193
28	$O^{\frac{1}{2}-}$	(a)	0·0121	0·035	0·8214
		(b)	0·0094	0·150	0·3171
29	Na^{+}	(a)	0·0081	0·418	0·1253
		(b)	0·0078	0·533	0·6376
$\frac{1}{16}$30	O	(a')	0·0247	0·938	0·1133
$\frac{15}{16}$		(a'')	0·0425	0·196	0·1137
		(b)	0·0375	0·188	0·6125
31	O	(a)	0·0218	0·910	0·9634
		(b)	0·0377	0·660	0·4589
$\frac{1}{32}$32	O	(a)	0·0183	0·331	0·0500
$\frac{31}{32}$32	O	(b)	0·0000	0·044	0·5000

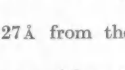
latest value of R is 26·4 % and on the $h0l$ term, with l even, 19·7 %. Some further improvement on these values should be possible, even with the present limited data, and further cycles of refinement are at present being carried out. In the meantime, it seems worth recording the general characteristics of the molecular and crystal structure found. These are illustrated by Fig. 5, which shows the electron-density peaks corresponding with the two cephalosporin C molecules as they appear in the 63-atom electron-density distribution. The atomic co-ordinates derived from this distribution are listed in Table 1. They define bond lengths which deviate by a maximum of 0·17 Å (in one case) and by an average of 0·07 Å from acceptable values for formula I. Such deviations are only to be expected at the present stage of structure refinement; it seems best to defer a full record of the bond lengths and bond angles in the molecule until refinement has been carried as far as possible.

Crystal and molecular structure of sodium cephalosporin C

In outline the crystal and molecular structure found for the sodium salt of cephalosporin C is very different from those found for sodium benzylpenicillin. Cephalosporin C, in Fig. 5, is shown in the correct absolute configuration, unlike benzylpenicillin in the first publication of the structure (Crowfoot, Bunn, Rogers-Low & Turner-Jones, 1949). Only the small region of the two molecules, including the β-lactam ring and the directly attached atoms, is closely similar. The amide chain in the cephalosporin C crystal is turned about the bond C-7–N-10, compared with that of benzylpenicillin, to a position with the amide carbonyl group nearly normal to the β-lactam ring. And the whole of the substituted adipic acid side chain is swung about the C-19–C-21 bond into a direction exactly opposite to that of the benzyl group in benzylpenicillin relative to the sulphur-containing ring. These changes in conformation may, of course, represent only temporary molecular situations which are stabilized in crystallization. They make possible a type of packing in sodium cephalosporin C reminiscent of many peptide crystals where a short-cell dimension of 4·8–4·9 Å corresponds with the hydrogen-bonded distance between parallel peptide chains. Here the hydrogen bond is between the amide  groups of successive molecules in the direction of the short b axis.

The six-membered sulphur-containing ring is rather flat. All of the atoms C-2, C-3, C-4, N-5, C-10 and C-15 lie in a plane within the limits of experimental error, with the sulphur atom about 0·6 Å above the plane and C-6 0·6 Å below it. This is in agreement with the formulation of C-3–C-4 as a double bond, which is also supported by its observed bond length of 1·31 Å. The changes in this part of the molecule make it possible for the attached carboxyl group here to lie much more nearly in the plane of the ring than it does in benzylpenicillin, where the carboxyl group is turned at about right angles to the five-membered ring.

The side chain at C-3 has the general form of an acetoxy group but is not very well defined. The four terminating atoms of the chain have an approximately planar configuration, but the last two are indistinguishable in electron density. This suggests that the two positions are occupied at random by oxygen and methyl groups corresponding to alternative positions reached by rotation about  . There appear to be no strong intermolecular forces in this region which might favour one packing arrangement rather than another.

The terminating atoms of the adipic acid side chain are, on the other hand, well defined. The two carboxyl oxygen atoms, by contacting succeeding Na^+ ions along the b axis, link the molecules together through ionic bonds. The amino group, by contrast with many amino acid structures, lies about 1·27 Å from the plane of the  system in a position at which it can form a hydrogen bond with the ring carboxyl group of a neighbouring molecule, O-16. The co-ordination sphere of the sodium ion is completed by contact with the second oxygen atoms, O-17 of this group and by two water molecules.

The differences that appear in the electron-density maps suggest that the two molecules, (*a*) and (*b*), lie at slightly different angles to the crystallographic b plane. This difference is probably to be correlated with the geometrical relation of the hydrogen bond between the amino group in one molecule and carboxyl group in another and the requirements of the sodium co-ordination sphere. Some modification of the detailed appearance of the atomic positions indicated in Fig. 5 must be expected on further refinement. But it seems unlikely that this could be sufficient to change our interpretation of the chemical structure of the molecule.

EXPERIMENTAL

X-ray photography

The b unit-cell dimension of cephalosporin C was measured on an oscillation photograph while d_{100}, d_{001} and β were determined on a Weissenberg photograph showing the $h0l$ reflexions together with lines from a copper specimen. The main series of reflexions were recorded on multiple film Weissenberg photographs taken with the b axis as rotation axis and copper K_α radiation. Three different crystals were used, of size $0{\cdot}6 \times 0{\cdot}25 \times 0{\cdot}1$ mm.^{3} for the zero and first layers, $1{\cdot}5 \times 0{\cdot}30 \times 0{\cdot}12$ mm.^{3} for the second and third layers, $1{\cdot}8 \times 0{\cdot}30 \times 0{\cdot}15$ mm.^{3} for the fourth layer. The intensities were first estimated visually by Miss G. Hurdman with some help from Miss B. Prestwood and entirely cross-checked by E.N.M. They were corrected for Lorentz, polarization and inclination factors by using a programme designed by C. K. Prout. Correlation between the layer lines was mainly effected by using a zero-layer Weissenberg photograph of a crystal rotating about the c axis. The intensities of reflexions in this film were measured both visually and by means of a double-beam microdensitometer. Correlation factors obtained from this film for the $h0l$, $h1l$, $h2l$ and $h3l$ reflexions were in good agreement with approximate values obtained from Wilson plots on each layer. $h4l$ reflexions did not appear on the c axis photograph; these were given similar scale and temperature factors to the other photographs. The whole of the data, placed on a common scale, were then set on an approximately absolute scale by means of a three-dimensional Wilson plot; B from this plot was 3·5.

Calculations

The main part of the calculations were carried out on the Ferranti Mercury automatic electronic computer at Oxford with a variety of programmes.

For the structure factor calculations the f curves calculated for S by Viervoll & Ögrim (1949) were employed, for Na^+ by Freeman (1959) and for O, N and C by Berghuis *et al.* (1955). The signs and phase angles calculated were not automatically carried over to the next electron-density calculation. In the early stages of projection analysis, a selection of terms where the calculated S contribution was above 0·5 was made. In the first three-dimensional series, ρ_1, hkl terms were only introduced where F_0 was above 0·04 and the calculated contribution of the four atoms above 0·25 of their possible maximum.

In addition, the $h0l$ terms were given the signs determined from the partly refined projection. At $\rho 4$–60 atoms, an attempt was made to derive the atomic positions precisely by a nineteen point routine analysis of the peaks in both $\rho 4$ and $\Delta\rho 4$ followed by an n shift where n was 1·75. Examination of the results of this calculation suggested that the peak positions at this stage were too poorly resolved for really satisfactory operation of the routine, particularly over the short bond C-3–C-4.

Interatomic distances were calculated, with programmes devised by Dr R. A. Sparks, at various stages of the analysis.

The full record of the intensity-data and structure-factor calculations preceding the evaluation of $\rho 5$ is given by Maslen (1960).

SUMMARY

1. Crystals of the sodium salt of cephalosporin C were grown slowly from water and ethanol and found to have a more complex monoclinic lattice than that earlier recorded, with

$$a = 38{\cdot}88 \pm 0{\cdot}15\text{Å}, \quad b = 4{\cdot}99 \pm 0{\cdot}04\text{Å},$$
$$c = 25{\cdot}65 \pm 0{\cdot}10\text{Å}, \quad \beta = 115° 25 \pm 15',$$

space group C2. The asymmetric unit contains two molecules of cephalosporin C, $C_{16}H_{22}O_8N_3SNa + 4$–5 molecules of water of crystallization.

2. The crystals were poorly reflecting. The intensities of all X-ray reflexions were measured visually out to the spacing limit observed about 1·04 Å.

3. From sharpened three-dimensional Patterson distributions, the positions of the sulphur atoms and of a number of other possible atoms in the crystal unit were found. These other atoms included five connected in a six-membered ring to the sulphur atom.

4. Further calculations, including electron-density distribution in two and three dimensions, enabled positions to be assigned to all the atoms in the unit cell. These positions cannot be given with great accuracy owing to the limited data available; they correspond closely with the requirements of formula (I) (p. 393).

5. Both in conformation and in molecular packing in the crystalline state sodium cephalosporin C is markedly different from sodium benzylpenicillin.

Our thanks are due to the Rhodes Trustees for a scholarship and to the National Research and Development Corporation for a grant to one of us (E.N.M.). We are also grateful for the assistance of the staff of the Oxford Computing laboratory in the calculations carried out and particularly to Dr J. S. Rollett, Dr R. A. Sparks and Dr C. K. Prout (Oxford) and Dr O. S. Mills (Manchester) for the use of their computer programmes. The main part of the intensity measurements were made by Miss G. Hurdman and technical assistance over the calculations was given by Mrs M. Innes.

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Derivatives of Cephalosporin C formed with certain Heterocyclic Tertiary Bases

THE CEPHALOSPORIN C_A FAMILY

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(*Received* 3 *August* 1960)

Cephalosporin C, an acidic antibiotic produced by a *Cephalosporium* sp., has been shown to have the structure (I) (Abraham & Newton, 1961; Hodgkin & Maslen, 1961). When a preparation of cephalosporin C which had been kept overnight in a pyridine–acetate buffer was chromatographed in the same buffer on an anion-exchange resin (Amberlite IR-4B), a substance which showed activity against *Staphylococcus aureus* was present in the first fractions from the column. This substance, named cephalosporin C_A, was well resolved from cephalosporin C itself. When the eluted cephalosporin C was concentrated and rechromatographed later under similar conditions, a further quantity of cephalosporin C_A appeared. It thus seemed that cephalosporin C_A was being formed from cephalosporin C in aqueous pyridine–acetate. Further experiments, which have been briefly reported by Abraham & Newton (1958) and by Hale, Abraham & Newton (1958), showed that the new active substance was the product of a reaction between cephalosporin C and pyridine.

When cephalosporin C was kept in 2M-pyridine–acetate at pH 7 and at temperatures varying from 37° to 70°, the activity of the solution against *Staph. aureus* rose to a value that was several-fold greater than the original one and then declined. Typical changes in activity are shown in Fig. 1. Electrophoresis on paper showed that this rise in activity was associated with the formation of a compound (cephalosporin C_A) that showed no net charge at pH 7·0. The compound was not formed when the pyridine buffer was replaced by the acetates of a number of other bases, including ammonia, trimethylamine, piperidine, or by pyridinium methyl bromide, aniline or *N*-dimethylaniline. It was formed more readily in aqueous pyridine at pH 7 than at pH 5 and its yield increased with the concentration of pyridine to a maximum at about 2M. Further work showed that a family of compounds could be produced by allowing cephalosporin C to react with different heterocyclic tertiary bases. The specific compound obtained with pyridine will be referred to as cephalosporin C_A (pyridine).

On a preparative scale, cephalosporin C_A (pyridine) was separated from remaining cephalosporin C, and from acidic degradation products formed during the reaction, by chromatography on Dowex 1X8 (acetate form). Cephalosporin C_A (pyridine) passed rapidly through the column and was obtained as an amorphous solid on freeze-drying the eluate. This product contained acetate and was extremely hygroscopic. For further purification it was adsorbed on Dowex 50X8 and eluted by stirring the resin with water and adding aqueous ammonia until the pH rose to 7·0. The resulting product was obtained as a non-hydroscopic white powder with an activity of approximately 120 u./mg. against *Staph. aureus* and

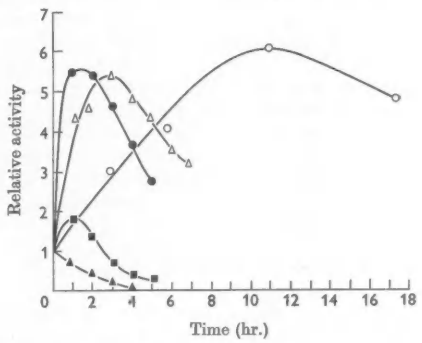

Fig. 1. Changes in activity against *Staph. aureus* with time, of solutions of cephalosporin C sodium salt (10 mg.) in 0·5 ml. of aq. pyridine–acetate (2M to pyridine), pH 7. ○, At 50°; △, at 65°; ●, at 70°; ■, at 80°; ▲, at 90°. Activities are given in arbitrary units.

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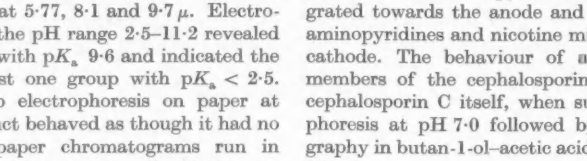
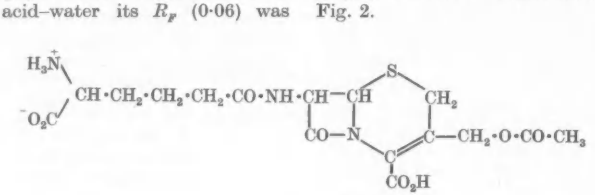
14 u./mg. against *Salmonella typhi* (cephalosporin C sodium salt has an activity of 8–10 u./mg. against both organisms).

Elementary analysis indicated that the product had the composition $C_{19}H_{22}O_6N_4S,3{\cdot}5H_2O$. The product yielded no *C*-methyl in the Kuhn–Roth determination. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ at 257 mμ ($E^{1\%}_{1cm.}$ 244). Its infrared-absorption spectrum showed a strong band at 5·67 μ, but no bands corresponding to those present in cephalosporin C at 5·77, 8·1 and 9·7 μ. Electrometric titration in the pH range 2·5–11·2 revealed an ionizable group with pK_a 9·6 and indicated the presence of at least one group with pK_a < 2·5. When subjected to electrophoresis on paper at pH 5 or 7 the product behaved as though it had no net charge. On paper chromatograms run in butan-1-ol–acetic acid–water its R_F (0·06) was lower than that (0·19) of cephalosporin C. Paper chromatography and electrophoresis indicated that it yielded α-aminoadipic acid on acid hydrolysis. When an acid hydrolysate was made alkaline and distilled, a volatile base was obtained in the distillate whose ultraviolet-absorption spectrum was indistinguishable from that of pyridine.

These properties suggested that cephalosporin C_A (pyridine) had the structure (II) and that its formation involved the replacement of the acetoxyl group in cephalosporin C by a pyridinium ion. Structure (II) was consistent with the N/S ratio of 4/1, the apparent formation of pyridine on hydrolysis, the absence of *C*-methyl, the absence of a band corresponding to an ester carbonyl (5·77 μ) in the infrared spectrum, and with the finding that the product showed no mobility on electrophoresis at pH 7. Alkylation of pyridine, though surprising, appeared feasible, in view of the fact that the acetoxy group formed part of an allylic structure. The pyridinium structure (II) received support from the mobility at pH 7·0 of other members of the cephalosporin C_A family which showed antibacterial activity (Table 1). Most of these compounds were not purified, but their behaviour on paper electrophoresis could be accounted for by the assumption that they were quaternary derivatives of the base used in their production. Thus the compounds formed from quinoline, 2:4:6-trimethylpyridine, 2-hydroxymethylpyridine and nicotinamide showed no net charge at pH 7·0, whereas those formed from pyridinecarboxylic acids migrated towards the anode and those formed from aminopyridines and nicotine migrated towards the cathode. The behaviour of a mixture of three members of the cephalosporin C_A family and of cephalosporin C itself, when subjected to electrophoresis at pH 7·0 followed by paper chromatography in butan-1-ol–acetic acid–water, is shown in Fig. 2.



(I)



(II)

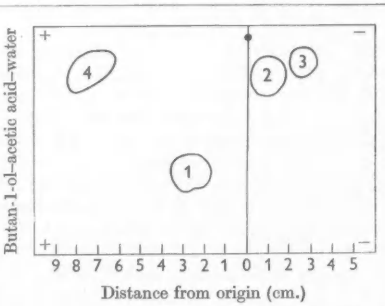


Fig. 2. Separation of cephalosporin C (1) and three members of the cephalosporin C_A family (2–4) by electrophoresis on paper at pH 7 (17 v/cm. for 4 hr.) followed by chromatography in butan-1-ol–acetic acid–water. A sample of the mixture was applied at ●. The cephalosporin C_A compounds were derived from cephalosporin C and pyridine (2), nicotine (3), pyridine-2:3-dicarboxylic acid (4). Spots were revealed by a bioautography with *Salm. typhi* as the test organism.

A compound formed from cephalosporin C and sulphapyridine migrated towards the anode, but less than half as far as cephalosporin C. This is consistent with the acidic nature of the sulphonamido group, which has pK_a 8·3 in sulphapyridine itself (Bell & Roblin, 1942) and would be expected to have a significantly lower pK_a when the pyridine nitrogen acquired a positive charge by conversion into a quaternary derivative. A compound obtained from sulphathiazole and cephalosporin C, whose formation presumably involved the positive charging of the nitrogen atom in the thiazole ring, also migrated towards the anode. The pK_a of the sulphonamido group in sulphathiazole is reported to be 7·12 (Bell & Roblin, 1942).

Further evidence for the quaternary nature of the members of the cephalosporin C_A family was provided by the properties of the compound obtained with nicotinamide. This compound was separated from unchanged cephalosporin C remaining in the reaction mixture by passage through a column of Dowex 1X10 (acetate form) and was obtained as an amorphous powder which showed an activity of 50 u./mg. against *Staph. aureus* and 12 u./mg. against *Salm. typhi*. Its ultraviolet-absorption spectrum in aqueous solution at pH 8 showed $\lambda_{max.}$ at 261 mμ with $E^{1\%}_{1\,cm.}$ 202. On the addition of sodium dithionite the $E_{261\,m\mu}$ fell to 151 and a broad band appeared with $\lambda_{max.}$ at 356mμ and $E^{1\%}_{1\,cm.}$ 96 (Fig. 3). The resulting solution showed a pale green–white fluorescence in ultraviolet light. When the pH of the solution was lowered to 2·0 the band in the ultraviolet-absorption spectrum with $\lambda_{max.}$ 356 mμ disappeared and a new band appeared with $\lambda_{max.}$ 293 mμ with $E^{1\%}_{1\,cm.}$ 218. This type of change in absorption spectrum is characteristic of quaternary nicotinamide derivatives, including the pyridine nucleotides (Warburg & Christian, 1936), the appearance of a band at about 350 mμ on reduction with sodium dithionite being

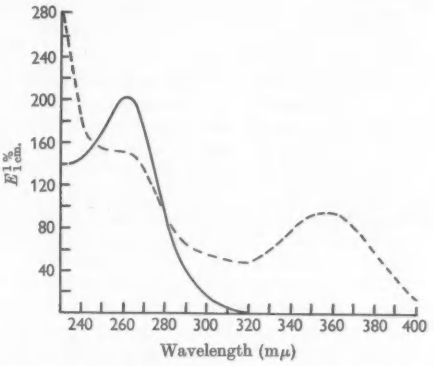


Fig. 3. Changes in the ultraviolet-absorption spectrum of cephalosporin C_A (nicotinamide) on reduction with sodium dithionite in 0·1 M-Na_2HPO_4. —, Before reduction; - - -, after reduction.

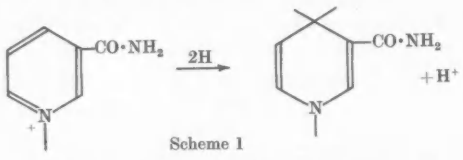
Table 1. *Electrophoretic mobility, R_F values and relative antibacterial activities of cephalosporin C_A derivatives*

Electrophoretic mobility is the distance travelled by the derivative relative to that travelled by cephalosporin C under similar conditions (pH 7·0 in collidine–acetate buffer; 17 v/cm.; 2·5 hr.). +, The derivative migrated towards the anode (negative charge at pH 7); −, migration towards the cathode. Substances with no net charge at pH 7·0 moved towards the cathode approximately 0·4 times the distance that cephalosporin C moved towards the anode. $R_{ceph\ C}$ represents the distances travelled by the derivatives relative to that travelled by cephalosporin C in butan-1-ol–acetic acid–water. Activity ratios represent the quotients [antibacterial activity against *Staph. aureus*/activity against *Salm. typhi*]. The quotient for cephalosporin C is assumed to be 1.

Cephalosporin C_A derived from:	Electrophoretic mobility	$R_{ceph\ C}$	Activity ratio
Nicotine	−1·4	0·16	5·0
2-Aminopyridine	−0·6	0·29	15·0
2-Amino-6-methylpyridine	−0·64	0·6	11·4
Pyridine	−0·4	0·31	10·0
Nicotinamide	−0·4	0·3	4·0
2:4:6-Trimethylpyridine	−0·4	0·54	3·9
2-Hydroxymethylpyridine	−0·4	0·36	3·6
Quinoline	−0·4	0·58	2·7
Sulphapyridine	+0·25	0·55	$\geqslant$1*
Sulphadiazine	+0·25	0·62	$\geqslant$1*
Sulphathiazole	+0·25	0·74	$\geqslant$1*
3-Hydroxypyridine	+0·75	0·36	5·0
*iso*Nicotinic acid	+0·55	0·2	1·30
Nicotinic acid	+1·0	0·23	0·94
Picolinic acid	+0·75	0·36	0·20
Pyridine-2:3-dicarboxylic acid	+3·2	0·33	0·05

* In these cases the inhibition zones on agar seeded with *Salm. typhi* were too small for the activity ratio to be estimated.

due to the reaction in Scheme 1 (Rafter & Colowick, 1954). After chromatography or electrophoresis on paper, cephalosporin C_A (nicotinamide) could be revealed by the procedure described by Kodicek & Reddi (1951) for the detection of quaternary nicotinamide derivatives.



Scheme 1

Different members of the cephalosporin C_A family varied greatly in their relative activities against *Staph. aureus* and *Salm. typhi*. Table 1 shows approximate values for the quotient [activity against *Staph. aureus*/activity against *Salm. typhi*] for a number of compounds, calculated on the assumption that the value for cephalosporin C itself is 1. The quotient was considerably increased when the acetoxy group of cephalosporin C was replaced by a quaternary aromatic base in which the substituents were either non-ionizable groups or groups (such as amino) whose ionization could provide an additional positive charge. This change tended to be reversed when the substituents were negatively charged carboxyl groups, and the nearer the carboxyl to the quaternary nitrogen the greater was its effect. Thus, the compound formed with *iso*nicotinic acid was slightly more active against *Staph. aureus* than against *Salm. typhi*, whereas that formed with picolinic acid was five times as active against *Salm. typhi* as against *Staph. aureus*, and that formed with pyridine-2:3-dicarboxylic acid was about 20 times as active against *Salm. typhi* as against *Staph. aureus*. It may be that these differences in activity are connected with differences in the facility with which the compounds reach their site of action in the two organisms.

EXPERIMENTAL

Antibacterial activities were measured by the hole-plate method (Brownlee *et al.* 1948) with *Staph. aureus* (N.C.T.C. 6571) and *Salm. typhi*, strain 'Mrs S' (Felix & Pitt, 1935) as test organisms and cephalosporin C sodium salt (8–10 μ/mg.) as a standard. Analyses were by Weiler and Strauss.

Paper chromatography and electrophoresis. Paper chromatograms were run on Whatman no. 1 paper in (*A*) butan-1-ol–acetic acid–water (4:1:4, by vol.) and (*B*) ethanol–water (7:3, v/v).

Electrophoresis was carried out on Whatman no. 1 paper at 17 v/cm. (for 2·5–4 hr. unless stated otherwise) in aq. collidine–acetate solution (0·05 M to acetate), pH 7·0, pyridine–acetate solution (0·05 M to acetate), pH 5·0, and in 10% (v/v) acetic acid, pH 2·2.

Members of the cephalosporin C_A family were seen as dark spots when the paper was placed before a source of ultraviolet light (230–400 mμ) and appeared as purple spots when the paper was sprayed with ninhydrin. They were also detected by means of bioautographs. The paper was placed on the surface of a plate of nutrient agar seeded with the test organisms. After 15 min. the paper was removed and the plate incubated overnight. Active compounds were located by clear areas where no bacterial growth had occurred. The compounds formed from cephalosporin C and sulphapyridine or sulphathiazole could also be detected by spraying the paper with 0·2% of $NaNO_2$ in 0·1N-HCl, drying at 60° and then spraying with 1% (w/v) α-naphthylamine in 75% (v/v) acetic acid. Aromatic amines give red spots under these conditions (Eckman, 1948). The compound formed from cephalosporin C and nicotinamide could be detected by suspending the paper for 1 hr. in the vapour of butan-2-one–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v). It then appeared as a blue–white fluorescent spot when the paper was viewed in ultraviolet light (365 mμ) (Kodicek & Reddi, 1951).

Small-scale production of members of the cephalosporin C_A family. Cephalosporin C sodium salt (10 mg.) was dissolved in an aqueous solution (0·50 ml.) of the base used. The concentration of the base was between 0·1 and 2·0 M and the pH of the solution was between 4·5 and 7·0, being adjusted (if necessary) to a value within this range by the addition of acetic acid. With sulphapyridine and sulphathiazole, whose solubility in water is low, the reaction was carried out in 50% (v/v) dimethylformamide. The mixture was kept at 37° and after different intervals (usually 24 and 48 hr.) 5μl. samples were spotted on paper for analysis by electrophoresis or chromatography or both. Bioautographs revealed the cephalosporin C_A and remaining cephalosporin C. Spraying with ninhydrin revealed cephalosporin C_A, cephalosporin C and usually also some 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (Jeffery, Abraham & Newton, 1960).

Estimations of relative antibacterial activities after paper chromatography or electrophoresis. Semi-quantitative estimates of the relative activities of members of the cephalosporin C_A family against *Staph. aureus* and *Salm. typhi* were obtained by the application of techniques similar to those used for the penicillins by Goodall & Levi (1947). A solution (10μl.) containing a mixture of cephalosporin C and a cephalosporin C_A compound was spotted on a strip of Whatman no. 1 paper in sixfold replication. The components were separated by chromatography or electrophoresis, and, after the solvent or buffer had been evaporated in a stream of air, the paper strip was applied to nutrient-agar sheets (30 cm. × 50 cm.) seeded with one of the test organisms. At the same time paper strips bearing standard spots of cephalosporin C in three graded amounts, each in sixfold replication, were applied to the sheet in such a way as to distribute the resulting inhibition zones at random about the agar sheet. After 15 min. the papers were removed and the agar sheets incubated overnight. The resulting inhibition zones were approximately elliptical and both axes were measured and their geometric means calculated. The mean diameters of the standard spots were plotted against the logarithm of the concentration. The relative activity of test spots was determined by applying their mean axes to the graph so formed. The accuracy of such a method was not high and assumed a

parallelism between log concentration/zone diameter for both cephalosporin C and the members of the C_A family which may not exist. However, it gave an indication of the relative activities of the derivatives against the two organisms, compared with the activity of cephalosporin C, before the derivatives had been isolated in quantity from reaction mixtures.

Preparation and purification of cephalosporin C_A (pyridine). A solution of cephalosporin C sodium salt (1 g.) in water (50 ml.) was brought to pH 2·5 by the addition of Dowex 50X8 (H form). About 1 g. of damp resin was required. The resin was removed by filtration and washed with a little water. Pyridine (8 ml.) was added to the combined filtrate and washing. The solution was kept at 46° for 18 hr. and then freeze-dried. The residue was dissolved in the minimum amount of water (about 2 ml.) and precipitated by the addition of acetone (about 50 ml.). The precipitate was centrifuged down and ground under dry acetone to form a pale buff-coloured powder. The powdered material was dissolved in water (about 2 ml.) and added to a column (9 cm. × 2 cm. diam.) of Dowex 1X10 (200–400 mesh; acetate form). Water was then allowed to flow through the column and the cephalosporin C_A (pyridine) emerged in the first 30 ml. of eluate. The latter was freeze-dried and the residue stirred with dry acetone. The resulting powder, freed from acetone *in vacuo*, weighed 250 mg. It showed an activity of 80 u./mg. against *Staph. aureus* and its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 257 mμ with $E^{1\%}_{1\,cm.}$ 180. The very hygroscopic product could be kept in a desiccator over P_2O_5.

A sample of this product (200 mg.) was dissolved in water (2 ml.) and the solution added to a column (5 cm. × 1 cm. diam.) of Dowex 50X8 (200–400 mesh; H form). The solution was applied to the column under slight air pressure and the column then washed rapidly with water (8 ml.). The resin was then removed from the column and stirred with water (about 25 ml.) while aq. NH_3 soln. was added [aq. NH_3 soln. (sp.gr. 0·88) diluted 1 to 4] until the pH rose to 6·9. The resin was filtered off and the clear filtrate freeze-dried. The residue was stirred with acetone, collected by centrifuging and dried *in vacuo* (118 mg.). This product was an almost white non-hygroscopic powder with an activity of 110–120 u./mg. against *Staph. aureus*. Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 257 mμ with $E^{1\%}_{1\,cm.}$ 244 (Found: C, 46·0; H, 6·2; N, 11·2; S, 5·8. $C_{19}H_{22}O_6N_4S,3{\cdot}5H_2O$ requires C, 46·0; H, 5·9; N, 11·2; S, 6·4%). When subjected to electrophoresis on paper at pH 5 or 7 it behaved as though it had no net charge; at pH 2·2 it migrated towards the cathode.

Preparation of cephalosporin C_A (nicotinamide). Cephalosporin C sodium salt (100 mg.) was dissolved in 5 ml. of water and Dowex 50X8 (H form) was added to the stirred solution until the pH fell to 2·6. (About 70 mg. of resin was required.) The resin was removed by filtration and washed with a little water. Nicotinamide (610 mg.) was dissolved in the combined filtrate and washing, when the pH rose to about 4·8. The solution was kept at 37° for 48 hr. and then freeze-dried. The residue was stirred three times with 10 ml. of acetone (to extract remaining nicotinamide) and the insoluble material separated each time by centrifuging. The dried material (74 mg.) was dissolved in about 1 ml. of water and added to a column (10 cm. × 1 cm. diam.) of Dowex 1X10 (200–400 mesh; acetate form). Water was allowed to pass through the column and 1 ml. fractions were collected. Fractions 3–6, which contained ninhydrin-positive material, were combined and freeze-dried. The product was stirred with 2 ml. of acetone, separated from the acetone by centrifuging, and dried *in vacuo* (28 mg.). It showed an activity of 50 u./mg. against *Staph. aureus* and 12 u./mg. against *Salm. typhi*. It gave a single spot (detected by bioautograph, ninhydrin, fluorescence with butan-2-one–NH_3 or absorption of ultraviolet light) when chromatographed on paper in system *A* (R_F 0·04), or in system *B* (R_F 0·43) and a single spot corresponding to a substance with no net charge when subjected to electrophoresis on paper at pH 7·0. It migrated 3 cm. towards the cathode on electrophoresis at pH 2·2.

Reduction with sodium dithionite. Cephalosporin C_A (nicotinamide) (1·15 mg.) was dissolved in 3·5 ml. of 0·1 M-Na_2HPO_4 (pH 8). A sample (0·5 ml.) of the solution was diluted to 3 ml. with 0·1 M-Na_2HPO_4 and the ultraviolet-absorption spectrum of the diluted solution was measured. The remainder of the original solution was bubbled through with N_2, 3 mg. of $Na_2S_2O_4$ was added, and the solution left in a closed tube for 50 min. The solution was then diluted to 9 ml. with 0·1 M-Na_2HPO_4, and O_2 was passed through it for 5 min. A sample was then diluted with an equal vol. of 0·1 M-Na_2HPO_4 and its ultraviolet-absorption spectrum measured (Fig. 3).

SUMMARY

1. Cephalosporin C reacts with pyridine in neutral aqueous solution to form a new compound with antibacterial activity. This compound shows no net charge on paper electrophoresis at pH 5–7. It has been named cephalosporin C_A (pyridine).

2. Cephalosporin C reacts in a similar fashion with a number of derivatives of pyridine, including nicotinamide, pyridinecarboxylic acids and sulphapyridine, and also with sulphathiazole and sulphadiazine. The resulting family of compounds has been named the cephalosporin C_A family.

3. Evidence is presented that the formation of members of the cephalosporin C_A family involves the displacement of the acetoxy group in cephalosporin by the heterocyclic tertiary base and conversion of the latter into a quaternary derivative.

4. The relative activity of members of the cephalosporin C_A family against *Staphylococcus aureus* and *Salmonella typhi* varies with the base from which they are formed. The compound formed with pyridine is at least 12 times as active as cephalosporin C against *Staphylococcus aureus* but less than twice as active against *Salmonella typhi*. The compound formed with pyridine-2:3-dicarboxylic acid is more than 20 times as active against *Salmonella typhi* as against *Staphylococcus aureus*.

We are indebted to Mrs M. Loveridge, Mr O. Boys, and Mr D. Gazzard for skilful technical assistance.

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The Cephalosporin C Nucleus (7-Aminocephalosporanic Acid) and some of its Derivatives

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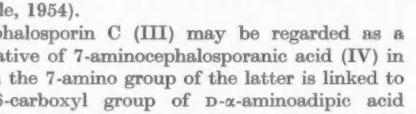
Cephalosporin N (I) (Newton & Abraham, 1954) may be regarded as a derivative of 6-aminopenicillanic acid (II) (Sheehan, Henery-Logan & Johnson, 1953) in which the 6-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid. Cephalosporin N has only about 1% of the antibacterial activity of benzylpenicillin against *Staphylococcus aureus* (Abraham, Newton & Hale, 1954).

Cephalosporin C (III) may be regarded as a derivative of 7-aminocephalosporanic acid (IV) in which the 7-amino group of the latter is linked to the δ-carboxyl group of D-α-aminoadipic acid (Abraham & Newton, 1961). Thus cephalosporin C and cephalosporin N have the same side chain, but different nuclei (II and IV respectively). Cephalosporin C has only about 10% of the activity of cephalosporin N against *Staph. aureus* (Newton & Abraham, 1956).

In view of these relationships it seemed probable that an analogue of cephalosporin C, which had a phenylacetyl group instead of a D-(δ-aminoadipoyl) group as a side chain, would be about 100 times as active as cephalosporin C and about one-tenth as active as benzylpenicillin against *Staph. aureus*. The preparation and *N*-acylation of 7-aminocephalosporanic acid provided one possible route to such an analogue.

In the course of work on the chemical structure of cephalosporin C, it became clear that this compound did not undergo a penicillin–penillic acid type of rearrangement and that its β-lactam ring was considerably more stable, under acid conditions, than the β-lactam ring of the penicillins.



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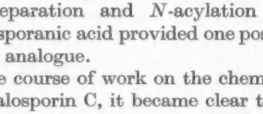

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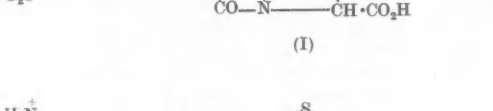

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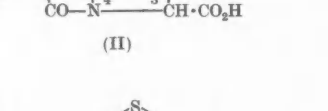
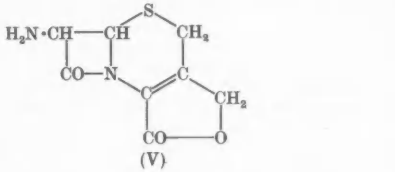


(IV)

This suggested that it might be possible to obtain the cephalosporin C nucleus (7-aminocephalosporanic acid, 7-ACA) by acid-catalysed removal of the side chain of cephalosporin C. The present paper reports evidence for the formation of the cephalosporin C nucleus, in small yield, and describes some of the properties of *N*-phenylacetyl and other *N*-acetyl derivatives of this nucleus. It also reports evidence for the formation of the nucleus of cephalosporin C_c (Abraham & Newton, 1961), which has been assigned the structure (V), and the nucleus of cephalosporin C_A (pyridine) (Hale, Newton & Abraham, 1961) which has been assigned the structure (VI).



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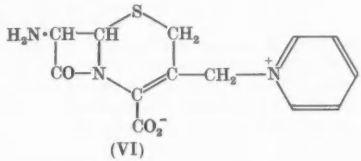


(VI)

After this work was projected, Batchelor, Doyle, Naylor & Rolinson (1959) reported the isolation of 6-aminopenicillanic acid (6-APA) from fermentations of *Penicillium chrysogenum* to which no side-chain precursor had been added. They found that 6-APA had only a weak antibacterial activity, but that it could be readily acylated to yield highly active penicillins. They also found that it had an isoelectric point of about 4·3. This low value for its isoelectric point is not surprising, since the 6-amino group is α to a lactam C=O and β to a thio ether grouping.

The structural relationship of 7-ACA and 6-APA suggested that the former would resemble the latter in having a very low antibacterial activity and a weakly basic amino group. We thus expected that 7-ACA would migrate as an acid on paper electrophoresis at pH 7, but not at pH 4, and that it would be detected on bioautographs only after acylation. The nucleus of cephalosporin C_c (Abraham & Newton, 1961) and that of cephalosporin C_A (pyridine) (Hale *et al.* 1961) were expected to be neutral at pH 7 and to migrate as bases at pH 4, since these two compounds would have one carboxyl group less and one strongly basic group more, respectively, than 7-ACA. The properties of certain products of acid hydrolysis of cephalosporin C, cephalosporin C_c and cephalosporin C_A (pyridine) corresponded so closely with these expectations that we became convinced that these products were, in fact, the desired compounds.

METHODS AND EXPERIMENTAL

Assay and unit of activity. Solutions were assayed by the hole-plate method with *Staph. aureus* (N.C.T.C. 6571) as the test organism. The unit is that used by Abraham *et al.* (1954). Cephalosporin C sodium salt has an activity of 8–10 u./mg.

Paper chromatography. Paper chromatograms were run at 20° on Whatman no. 1 paper in the following solvent systems: (*A*) butan-1-ol–ethanol–water (4:1:5, by vol.) (Batchelor *et al.* 1959); (*B*) ethyl acetate saturated with aqueous sodium acetate buffer (0·1 M to Na), pH 5·2, with paper pretreated with the buffer (Hale, Miller & Kelly, 1953). The paper was soaked in the buffer, blotted, hung in a stream of air at room temperature, and used as soon as dry. With this system the solvent front reached the bottom of the paper in about 3 hr. but the solvent was allowed to run for 18 hr.

Paper electrophoresis. Paper electrophoresis on Whatman no. 1 paper (14 v/cm., for 3 hr. unless stated otherwise) was carried out as described by Newton & Abraham (1954). The following buffer systems were used: collidine–acetate (0·05 M to acetic acid), pH 7·0; pyridine–acetate (0·05 M to acetic acid), pH 4·0 or 4·5; 10% (v/v) acetic acid, pH 2·2; 10% (v/v) formic acid, pH 1·5.

Bioautographs. These were prepared as described by Hale *et al.* (1961).

Treatment with penicillinase on paper. In some cases spots of compounds on paper were sprayed with a solution of penicillinase in 1% (w/v) gelatin before the paper was used for the preparation of a bioautograph. The penicillinase used was a highly purified preparation of the induced enzyme from *Bacillus cereus* 569, kindly provided by Dr M. R. Pollock.

N-*Acylation of compounds on Whatman no.* 1 *paper.* Cephalosporin C, 7-ACA and related compounds were converted into their *N*-phenylacetyl derivatives on paper by a modification of the method of Batchelor *et al.* (1959). After paper chromatography or paper electrophoresis, the papers were dried at room temperature in a current of air. They were sprayed evenly with M-pyridine in 50% (v/v) acetone until they were barely damp. They were then lightly sprayed with 2% (w/v) phenylacetyl chloride in acetone, and again with the pyridine solution until a spot of bromocresol green, which had been placed on the paper, immediately turned blue (pH 5·0). After hanging in an air stream for 3–5 min. the papers were contacted with plates seeded with *S. aureus* (Oxford strain N.C.T.C. 6571). Other *N*-acetyl derivatives were prepared on paper and detected in a similar manner.

N-*Acylation of cephalosporin C and of its derivatives in solution* (*a*). A solution of cephalosporin C or one of its derivatives (1 equiv.) was neutralized (pH 5–6), if necessary, with $NaHCO_3$ solution. Further $NaHCO_3$ solution (4 equiv), which had been gassed with CO_2 to pH 7·0, was added to the solution. At this stage the concentration of the compound to be acylated was about 2% (w/v). Acetone (0·5 vol.) was then added, and the mixture was cooled to 0°.

Phenylacetyl chloride (1·2 equiv.), in 0·5 vol. of acetone, was added to the cooled stirred solution through a capillary over a period of 20 min. The pH of the mixture was maintained at 7·9 (glass electrode) by passing over it a slow stream of CO_2. After the phenylacetyl chloride had been added, the flask was corked, and the mixture was stirred at 0° for 20 min. and at room temperature for a further 30 min. The pH of the solution was checked at 10 min. intervals, and more CO_2 passed into the flask if necessary. Excess of bicarbonate was then neutralized with HCl (pH 5·5–6·0, glass electrode), and the acetone removed *in vacuo*. The pH of the aqueous residue was readjusted to 5–6.

(*b*) Small-scale acylations were carried out in aqueous pyridine. The compound to be acylated (4 mg.) was dissolved in a mixture of water (42μl.) and pyridine (8μl.). To a 10μl. sample of this was added a solution of the acid chloride (1·5 mol.prop.) in acetone (10μl.). The mixture was kept for 15 min. at room temperature and samples (5–10μl.) of the mixture (or of the mixture diluted with water if necessary to yield suitable inhibition zones on bioautographs) were applied directly to paper for electrophoresis or chromatography.

Extraction of N-acyl derivatives into butan-1-ol or n-butyl acetate. The *N*-phenylacetyl derivatives of cephalosporin C, made by procedure (*a*), were extracted into butan-1-ol at pH 2·2, and re-extracted into aqueous solution at pH 6·5 in the manner described by Newton & Abraham (1954). The *N*-phenylacetyl derivative of 7-ACA made by procedure (*a*), was extracted in a similar manner into *n*-butyl acetate at pH 1·8 and re-extracted into aqueous solution at pH 5·0. The resulting aqueous solutions of the sodium salts were freeze-dried.

Reaction of 7-aminocephalosporanic acid and its N-acyl derivatives with pyridine on paper. The conversion of 7-ACA into the cephalosporin C_A (pyridine) nucleus and of *N*-acyl derivatives of 7-ACA into compounds of the C_A type could be demonstrated on paper. A strip of Whatman no. 1 paper, containing a spot of the substance studied (in sufficient concentration to give an inhibition zone of convenient size for a final bioautograph) was hung in a closed vessel for 18 hr. at 37° above the surface of aqueous 2M-pyridine adjusted to pH 7·0 with acetic acid. The spot was then subjected to electrophoresis or chromatography on the same paper. For 7-ACA the paper was sprayed with pyridine in 50% (v/v) acetone and then phenylacetyl chloride in acetone before bioautography.

Investigation of conditions for the hydrolysis of cephalosporin C and its derivatives with acid. Cephalosporin C free acid or cephalosporin C_c (50 mg.) (Abraham & Newton, 1961) was dissolved in 1·3 ml. of a solution of the acid used. The mixture was heated in a temperature-controlled bath at 20°, and samples (5μl.) were removed at selected times for analysis directly by paper chromatography or by paper electrophoresis or both. Hydrolysis of cephalosporin C_A (pyridine) was carried out similarly, but 1·7μl. samples of the resulting solutions were used for analysis.

Hydrolysis of cephalosporin C free acid (50 mg. in 1·3 ml. of water) was also carried out with various amounts (20–500 mg.) of Dowex 50X8 (H form; 200–400 mesh). At equilibrium the pH of the solution varied from 2·8 to 2·4. After the mixture had been kept at 20° for a given time (without stirring), its pH was brought to 5·0 with 8% (w/v) $NaHCO_3$ solution, and then to 6·5 with N-NaOH. The solution was freed from the resin and a sample (5μl.) was analysed by paper electrophoresis.

Production of crude 7-aminocephalosporanic acid. Cephalosporin C sodium salt (2 g.) was dissolved in 30 ml. of water, the pH adjusted to 2·5 by addition of Dowex 50X8 (H form; 200–400 mesh) (about 2 g. of damp resin), and the resin filtered and washed with 10 ml. water. To the combined filtrate and washings, containing cephalosporin C free acid, was added 10·2 ml. of N-HCl. The solution was kept at 20° for 3 days and then added to a column (2.1 cm. diam. × 7 cm.) of Dowex 1X8 (200–400 mesh; acetate form). The eluate was collected in 5 ml. fractions (1–12) and the column was eluted with water until a total of 34 fractions had been collected. Elution was then begun with 0·5N-acetic acid and a further 66 fractions were collected. The extinction at 260 mμ was measured for each fraction.

Fractions 2–16 were pooled and concentrated *in vacuo*, when cephalosporin C_c (312 mg.) (Abraham & Newton, 1961) separated in crystalline form. Fractions 36–45 contained most of the 7-ACA as revealed by the formation of an active derivative when ten 10μl. spots from each fraction were phenylacetylated on paper. These fractions were pooled and freeze-dried to yield a white powder (40 mg.). On phenylacetylation by procedure (*a*) 1 mg. of this material yielded 250 units of activity against *Staph. aureus*.

Purification of crude 7-aminocephalosporanic acid by paper electrophoresis. The crude 7-ACA obtained from the Dowex 1 (acetate) column (35 mg.) was further purified by electrophoresis in a Beckman Spinco Model CP continuous-flow paper-electrophoresis cell. The buffer used was made by adding pyridine to 0·05 N-acetic acid until the pH rose to 4·0. The cell was run at constant current (40 mA), the potential being 880 V. The sample, in 20 ml. of buffer, was fed to the curtain during 24 hr. and 32 fractions were collected, the fractions being numbered from cathode (1) to anode (32). The volume of each fraction was about 12 ml. At the end of the experiment the paper curtain was sprayed with ninhydrin. This revealed a strong band of material with very little mobility which flowed off the curtain in fractions 13–15, a band of strongly acidic material, which had flowed into the anode wick, and a faint band of a product (corresponding to α-aminoadipic acid) which had migrated towards the anode and flowed off the curtain mainly in fraction 24.

Fractions 22 and 23 contained the 7-ACA as revealed by the formation of an active derivative when ten 10μl. spots were phenylacetylated on paper.

Freeze-drying of fraction 22 yielded a residue which was too small to be weighed accurately. In aqueous solution the ultraviolet-absorption spectrum of the product showed a plateau at 260 mμ, and its total weight (43μg.) was estimated from the extinction at this wavelength on the assumption that the molecular weight of the substance was that of 7-ACA and that its molecular extinction was the same as that of cephalosporin C.

The product was dissolved in a mixture of 1 ml. of water and 0·5 ml. of 0·25 M-sodium phosphate buffer, pH 6·5. The solution was cooled to 0° and 1 ml. of a 4% (w/v) solution of phenylacetyl chloride in acetone was added slowly, with stirring, during 20 min. The mixture was kept for a further 20 min. at 0° and subsequently for 30 min. at room temperature. It was then diluted to 5 ml. with water and assayed against *Staph. aureus*. The assay indicated that 1 mg. of the product yielded 1450 units of activity on phenylacetylation.

Table 1. *Results from bioautographs prepared after electrophoresis*

Electrophoresis was at 14 v/cm. (except formic acid 10 v/cm.) for 3 hr. Distance travelled from origin is expressed as + or − to indicate movements to anode or cathode. Glucose was revealed by spraying with 2% (w/v) aniline hydrogen phthalate in butanol-saturated water, and leucine, α-aminoadipic acid and glycine by ninhydrin.

	Distance travelled (cm.) from origin in buffer				
	Collidine–acetate	Pyridine–acetate		Formic acid	Acetic acid
Compound	pH 7·0	pH 4·5	pH 4·0	pH 1·5	pH 2·2
Cephalosporin C	+5·2	+7·5	+7·2	−3·2	−0·4
N-Phenylacetylcephalosporin C	+8·2	+12·0	—	—	—
Cephalosporin C_c	−1·5	−2·0	—	−4·0	−5·0
N-Phenylacetylcephalosporin C_c	+3·4	—	—	—	—
Cephalosporin C_A (pyridine)	−1·5	—	−2·9	—	−4·7
Cephalosporin C_A (pyridine) nucleus	−1·5	—	−9·8	—	−9·9
Cephalosporin C_c nucleus	−1·5	−4·5	—	—	−13·1
Penicillin nucleus (6-APA)	+7·5	+2·1	0·0	−6·5	−10·0
Cephalosporin C nucleus (7-ACA)	+7·5	+3·4	+1·0	−4·5	−4·0
N-Phenylacetyl derivative of 6-APA (benzylpenicillin)	+5·2	+7·5	—	—	—
N-Phenylacetyl derivative of 7-ACA	+5·1	+7·5	—	—	—
Glycine	−1·5	—	—	−10·2	−14·1
Leucine	−1·5	−2·0	—	−7·4	−10·7
α-Aminoadipic acid	—	+4·0	—	−6·0	−7·8
Glucose	−1·5	−2·0	—	—	−1·0

(Cephalosporin C sodium salt shows an activity of 8–10 u./mg.) On the basis of this value the overall yield of 7-ACA obtained after chromatography on Dowex 1 was 0·6% of that theoretically possible. However, the overall yield of 7-ACA isolated after continuous paper electrophoresis was only about 0·006%. Hence, a large proportion of the product was lost during the latter process.

Preparations of 7-ACA obtained in this way still contained a small amount of α-aminoadipic acid. They could be freed from α-aminoadipic acid by electrophoresis on paper in 10% (v/v) acetic acid, pH 2·2, and elution from the paper with 0·1N-acetic acid. Under these conditions of electrophoresis 7-ACA migrated towards the cathode about half as fast as α-aminoadipic acid.

RESULTS

Products of hydrolysis of cephalosporin C and cephalosporin C_c. Bioautographs, made after paper electrophoresis (Table 1) and paper chromatography (Table 2), of solutions obtained by keeping cephalosporin C free acid in dilute HCl at 20° showed the presence of two active compounds. When the paper was sprayed with phenylacetyl chloride two further active compounds were revealed. The first two compounds corresponded with unchanged cephalosporin C and cephalosporin C_c. The third had the properties expected of 7-ACA and the fourth had the properties expected of the nucleus of cephalosporin C_c. The fourth compound was indistinguishable from a compound found to be present in hydrolysates of cephalosporin C_c.

Products of hydrolysis of cephalosporin C_A (pyridine). Hydrolysates obtained by treating

Table 2. *Results from bioautographs prepared from paper chromatograms*

Paper chromatograms were run with butan-1-ol–ethanol–water (4:1:5, by vol.).

Compound	R_F
Cephalosporin C	0·04
N-Phenylacetylcephalosporin C	0·13
Cephalosporin C_c	0·09
N-Phenylacetylcephalosporin C_c	0·23
Cephalosporin C_A (pyridine)	0·00
Cephalosporin C_A (pyridine) nucleus	0·07
Cephalosporin C_c nucleus	0·26
Penicillin nucleus (6-APA)	0·14
Cephalosporin C nucleus (7-ACA)	0·14
N-Phenylacetyl derivative of 6-APA (benzylpenicillin)	0·45
N-Phenylacetyl derivative of 7-ACA	0·40

cephalosporin C_A (pyridine) with dilute HCl at 20° contained unchanged cephalosporin C_A and a second compound whose activity was greatly increased by phenylacetylation. The second compound had the properties expected of the nucleus of cephalosporin C_A (pyridine).

Variation in the composition of hydrolysates of cephalosporin C and cephalosporin C_A with experimental conditions. Table 3 indicates that, for a given concentration of aqueous acid, the concentration of cephalosporin C gradually declines, and that 7-ACA, cephalosporin C_c, and cephalosporin C_c nucleus successively reach a maximum concentration. The amounts of 7-ACA and cephalosporin C_c then decline in turn. Table 4 indicates that, when hydrolysis was catalysed by Dowex 50 (H form),

Table 3. *Products of hydrolysis of cephalosporin C and cephalosporin C_c*

Hydrolysis was carried out at 20° with various amounts of HCl or, in one case, toluene-*p*-sulphonic acid (for details see Methods). The amount of acid used is stated in terms of equiv./mole of cephalosporin C. Samples of the hydrolysate were subjected to paper electrophoresis or chromatography to separate products of hydrolysis, and the papers then sprayed with a solution of phenylacetyl chloride (for details see Methods). Bioautographs were prepared (after phenylacetylation) with *Staph. aureus* as the test organism. The number of crosses gives an indication of the relative sizes of inhibition zones.

Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivative of			
Substance	HCl (equiv./mole)	Time (hr.)	Cephalosporin C	7-ACA	Cephalosporin C_c	Cephalosporin C_c nucleus
Cephalosporin C	1·1	24	+ + + +	+	+	−
		48	+ + +	+ +	+	−
		72	+ + +	+ +	+ +	−
		96	+ +	+ +	+ +	−
		120	+	+	+ + +	−
	2·1	16	+ + + +	+ +	+	−
		42	+ + +	+ + +	+	+
		72	+ +	+ + + +	+ +	+
		90	+	+ +	+ + +	+ +
	3·1	42	+	+ +	+ + +	+ +
		72	−	+	+ + +	+ +
		90	−	±	+ + +	+ +
	4·1	42	±	+	+ + +	+ + +
		72	−	±	+ + +	+ + +
		90	−	−	+ +	+ + +
	5·5	16	+ +	+ + +	+ +	+ +
		40	±	+	+ + +	+ + +
		64	−	±	+ +	+ + +
	11·0	18	+ +	+ +	+ + +	+ + +
		24	+	+ +	+ +	+ + +
		40	−	±	+	+ + + +
		72	−	−	+	+ + + +
	1·2*	15	+ + + +	+	+	−
		87	+ + +	+ + +	+ +	−
Cephalosporin C_c	1·1	42	.	.	+ + + + +	−
	6·0	18	.	.	+ + +	+ + +
		90	.	.	+ +	+ + +
	11·0	18	.	.	+ + +	+ + + +

* Toluene-*p*-sulphonic acid.

Table 4. *Products of hydrolysis of cephalosporin C*

Hydrolysis was carried out at 20° with Dowex 50X8 (200–400 mesh; H form) (for details see Methods). The amount of Dowex 50 used is stated in terms of mg. of damp resin/50 mg. of cephalosporin C. The pH values of the solutions at equilibrium varied from 2·8 to 2·4. Hydrolysates were analysed as described for Table 3.

Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivative of			
Amount of resin (mg.)	Time (hr.)	Cephalosporin C	7-ACA	Cephalosporin C_c	Cephalosporin C_c nucleus
20	46	+ + + +	+	+	+
40	46	+ + +	+ +	+ +	+ +
100	15	+ +	−	+ +	+ + +
	46	+	−	−	+ + +
500	36	−	−	−	+ + +

Table 5. *Products of hydrolysis of cephalosporin C_A (pyridine)*

Hydrolysis was carried out at 20° with various amounts of HCl (for details see Methods). The amount of acid used is stated in terms of equiv./mole of cephalosporin C_A. Hydrolysates were analysed as described for Table 3.

Conditions of hydrolysis		Size of inhibition zone on bioautograph attributed to phenylacetyl derivatives of	
HCl (equiv.)	Time (hr.)	Cephalosporin C_A (pyridine)	Cephalosporin C_A (pyridine) nucleus
1·2	72	+ + + + + + + +	+ + + + + + + +
	400	+ + + + + +	+ + + + + +
11	16	+ + + + + +	+ + + + + +
	72	+ + + + + +	+ + + + + +
	400	+ + +	+ + + + +

cephalosporin C_c nucleus was formed much more rapidly than in a solution of HCl having a similar pH. Table 5, when compared with Table 3, indicates that cephalosporin C_A (pyridine) and cephalosporin C_A (pyridine) nucleus were considerably more stable in aqueous acid than cephalosporin C and 7-ACA respectively.

Estimations of the activity of solutions obtained by hydrolysing cephalosporin C for 72 hr. with 2·1 equiv. of acid (see Table 3) were made by the hole-plate method before and after phenylacetylation of the solutions. Before phenylacetylation 20% of the original activity remained. Bioautographs made after paper electrophoresis indicated that about half of this activity was due to unchanged cephalosporin C and half to cephalosporin C_c. After phenylacetylation the activity of the hydrolysate was about 125% of that of the original solution of cephalosporin C. Phenylacetylation of cephalosporin C and cephalosporin C_c under similar conditions resulted in an approximately twofold increase in activity. Hence the total activity of the phenylacetyl derivative of the 7-ACA formed on hydrolysis was about 85% of that of the original cephalosporin C. On the assumption that 1 mg. of 7-ACA gives 1450 units of activity on phenylacetylation (see Experimental section) the amount of 7-ACA formed under the conditions used was 0·93% of that theoretically possible.

Properties of 7-aminocephalosporanic acid in relation to those of 6-aminopenicillanic acid

A sample (50 μg.) of 7-ACA that had been finally purified by electrophoresis on paper at pH 2·2 yielded no detectable amount of α-aminoadipic acid on hydrolysis with N-HCl at 105° for 16 hr. Paper chromatography of the hydrolysate showed a weak ninhydrin-positive spot, corresponding to glycine, which was similar in intensity to a spot in the same position obtained from a hydrolysate of 50 μg. of cephalosporin C.

With ninhydrin 7-ACA gave a brownish-yellow colour on paper, similar to that given by 6-APA. In both cases the colour later changed to a grey–blue. Unlike 6-APA, however, 7-ACA could be detected on paper as a dark (light-absorbing) spot by placing the paper before a source of ultraviolet light (230–400 mμ). The inhibition zone obtained on a bioautograph after 7-ACA had been phenylacetylated on paper was undiminished when the spot of 7-ACA was first sprayed with a solution of purified penicillinase in ten times the concentration required to inactivate a similar amount of benzylpenicillin.

7-ACA migrated towards the anode at almost the same rate as 6-APA, but faster than cephalosporin C, on paper electrophoresis at pH 7·0. At pH 4·5 it migrated about half as fast as cephalosporin C and somewhat faster than 6-APA. At pH 4·0 both 7-ACA and 6-APA showed only a small mobility towards the anode, but the former moved significantly further than the latter. On paper electrophoresis at pH 2·2, 7-ACA moved towards the cathode at about half the rate of 6-APA or α-aminoadipic acid, and less rapidly than aspartic acid. At pH 1·5, however, 7-ACA migrated 0·7 times as fast towards the cathode as 6-APA.

On paper chromatography in butan-1-ol–ethanol–water, 7-ACA, 6-APA, and *N*-phenylacetyl cephalosporin C showed very similar R_F values.

7-ACA, unlike 6-APA, was partly transformed, when exposed on paper to the vapour of 2M-pyridine–acetate, pH 7, to a compound that showed no net charge on electrophoresis at pH 7·0. The new compound was detected by its activity after phenylacetylation on paper. It was indistinguishable, on paper electrophoresis at pH values between 2·2 and 7·0 and on paper chromatography in butan-1-ol–ethanol–water, from the product of acid hydrolysis of cephalosporin C_A (pyridine) that was assumed to be the cephalosporin C_A (pyridine) nucleus.

N-Acyl derivatives of 7-aminocephalosporanic acid

Spots of 7-ACA on paper were converted by treatment with a number of different acid chlorides into derivatives which showed activity against

Staph. aureus. The sizes of inhibition zones on bioautographs suggested that the activity of the phenoxyacetyl derivative was similar to that of the phenylacetyl derivative but that the activities of the benzoyl, *iso*butyryl, propionyl and acetyl derivatives were of a lower order. All these derivatives migrated towards the anode at a rate similar to that of cephalosporin C when subjected to electrophoresis on paper at pH 4·5 or pH 7·0. The phenylacetyl derivative was readily extractable into butyl acetate from an aqueous solution at pH 1·8.

The phenylacetyl, phenoxyacetyl, and propionyl derivatives of 7-ACA were separated from each other, and from the corresponding derivatives of 6-APA, when chromatographed on paper in an ethyl acetate–sodium acetate buffer system. The R_F values of the different compounds relative to that of benzylpenicillin (the *N*-phenylacetyl derivative of 6-APA) are given in Table 6. The R_F values of both series of derivatives increase in the order propionyl, phenylacetyl, phenoxyacetyl to a similar relative extent, but the R_F of each derivative of 7-ACA is considerably lower than that of the corresponding derivative of 6-APA.

Spots on paper of the phenylacetyl and phenoxyacetyl derivatives of 7-ACA were exposed to the vapour of aqueous pyridine–acetate at 37° for 24 hr. Electrophoresis and bioautography (with *Staph. aureus* as the test organism) then revealed the presence of two compounds which showed no net charge at pH 7·0, in addition to the original derivatives. A similar result was obtained when the phenylacetyl derivative of 7-ACA was kept in 2 M-pyridine (pH 7) for 48 hr. at 37° and then subjected to electrophoresis. It thus appeared that the derivatives had been partly transformed to pyridinium compounds of the cephalosporin C_A type. However, the areas of the inhibition zones produced by the new compounds of the C_A type were about 0·3 times as large as those produced by the remaining phenylacetyl and phenoxyacetyl derivatives of 7-ACA. In comparable experiments with cephalosporin C the inhibition zone produced by cephalosporin C_A was about twice as large as that produced by the remaining parent compound.

Under the conditions used no inhibition zones were produced by the phenylacetyl derivative of 7-ACA, or by the C_A compound formed from it, when attempts were made to obtain bioautographs with *Salmonella typhi* as the test organism. With *Staph. aureus* as the test organism the size of the inhibition zone produced by a spot on paper of about 10 μg. of the phenylacetyl derivative of 7-ACA was not significantly changed when the paper was sprayed, before testing, with a solution of penicillinase which completely inactivated a 50 μg. spot of benzylpenicillin.

Table 6. *Comparison of derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid on paper chromatography*

Chromatograms were run in an ethyl acetate–sodium acetate buffer system, pH 5·2 (for details see Methods). Spots were revealed on bioautographs, *Staph. aureus* being the test organism.

Derivative	$R_{\text{benzylpenicillin}}$ for derivative of 7-ACA	$R_{\text{benzylpenicillin}}$ for derivative of 6-APA
Propionyl	0·18	0·43
Phenylacetyl	0·46	1·00
Phenoxyacetyl	0·51	1·07
α-Phenoxypropionyl	0·78	1·28

Properties of the cephalosporin C_c nucleus and cephalosporin C_A (pyridine) nucleus

The cephalosporin C_c nucleus and the cephalosporin C_A (pyridine) nucleus behaved as though they had no net charge when subjected to electrophoresis on paper at pH 7·0, but migrated towards the cathode at pH 4·5, pH 4·0 and pH 2.2. At pH 7·0 they were not resolved from each other, but at pH 2·2 the cephalosporin C_c nucleus migrated further towards the cathode than the cephalosporin C_A nucleus. The compounds were readily distinguished on chromatograms run with butan-1-ol–ethanol–water.

The activity of the cephalosporin C_c nucleus and the cephalosporin C_A (pyridine) nucleus (formed from 400 μg. and 40 μg. respectively of cephalosporin C) was not affected significantly, on paper, by a solution of penicillinase which completely destroyed 50 μg. of benzylpenicillin under similar conditions.

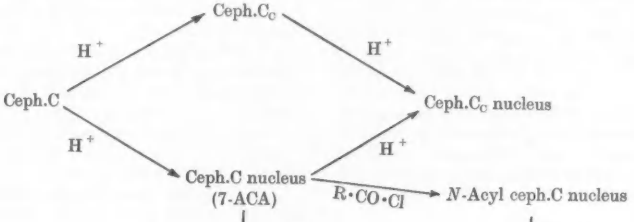
The cephalosporin C_c nucleus did not appear to react with pyridine to give a derivative of the C_A type.

DISCUSSION

Some of the products that are formed when cephalosporin C or cephalosporin C_A (pyridine) is treated with dilute acid at room temperature appear to be related according to Scheme I (p. 415). The cephalosporin C_c nucleus is a relatively stable end product than can be formed either from 7-ACA or from cephalosporin C_c. The cephalosporin C_A nucleus can be formed from 7-ACA by reaction with pyridine or from cephalosporin C_A by hydrolysis.

The yield of 7-ACA, at least, was very small under the conditions used and the formation of this compound would have been difficult to detect, had it not given a highly active *N*-phenylacetyl derivative. The susceptibility of the acetoxy group in both cephalosporin C and 7-ACA to acid-cata-

lysed displacement places a serious obstacle in the way of attempts, by methods of the type described in this paper, to obtain substantial amounts of 7-ACA from cephalosporin C. It is possible that these methods would be satisfactory, however, for the preparation of the nucleus of cephalosporin C_c and that of cephalosporin C_A.



Scheme I

Preliminary experiments on the purification of 7-ACA gave a product whose *N*-phenylacetyl derivative was about 100 times as active as cephalosporin C against *Staph. aureus*. This product yielded no α-aminoadipic acid on hydrolysis and showed, like 6-APA, an atypical ninhydrin reaction on paper. Neither 7-ACA, nor the nucleus of cephalosporin C_c or of cephalosporin C_A, was isolated in crystalline form. Nevertheless, the properties of these products and their acyl derivatives, as revealed by paper electrophoresis, paper chromatography and bioautography, formed a self-consistent picture which could not be accounted for in terms of alternative structures. The hypothesis was considered that the substance now assumed to be 7-ACA was the corresponding deacetyl compound, but this was rejected on two counts. First, the substance reacted with pyridine to form a derivative of the cephalosporin C_A type; cephalosporin C reacts with pyridine in this way (Hale *et al.* 1961), but deacetylcephalosporin C does not (J. d'A. Jeffery, G. G. F. Newton & E. P. Abraham, unpublished work). Secondly, the substance was formed under acid conditions in which, by analogy with deacetylcephalosporin C, deacetyl-7-ACA would be expected to undergo rapid lactonization to the nucleus of cephalosporin C_c (J. d'A. Jeffery, G. G. F. Newton & E. P. Abraham, unpublished work).

In a number of its properties 7-ACA showed a close similarity to 6-APA. This resemblance extended to the *N*-acetyl derivative of the two compounds. When chromatographed on paper in an ethyl acetate–sodium acetate buffer system the *N*-acyl derivatives of 7-ACA showed R_F values which increased in the order, propionyl, phenylacetyl and phenoxyacetyl, and the size of inhibition zones indicated that the activity of the *N*-propionyl and *N*-benzoyl derivatives against *Staph. aureus* was much less than that of the *N*-phenylacetyl and *N*-phenoxyacetyl derivatives. Similar relationships were obtained with the corresponding *N*-acyl derivatives of 6-APA. However, the behaviour of 7-ACA and 6-APA on paper electrophoresis at pH values between 4·5 and 2·2 indicated that the carboxyl group of the former was significantly stronger than that of the latter. This difference in the pK_a of the carboxyl group may be partly responsible for the fact that the R_F values of *N*-acyl derivatives of 7-ACA in ethyl acetate–sodium acetate buffer were considerably lower than the R_F values of the corresponding derivatives of 6-APA. The *N*-phenylacetyl derivative of 7-ACA also differed from the corresponding derivative of 6-APA (benzylpenicillin) in being relatively insensitive to purified penicillinase from *B. cereus*. Stability to penicillinase is therefore not destroyed when the δ-(α-aminoadipoyl) side chain of cephalosporin C is exchanged for a non-polar grouping.

SUMMARY

1. Treatment of cephalosporin C with dilute acid, at room temperature, has produced, in small yield, a compound whose properties correspond with those of the cephalosporin C nucleus (7-aminocephalosporanic acid). This compound is formed by the hydrolytic removal of the D-(δ-aminoadipoyl) side chain of cephalosporin C.

2. 7-Aminocephalosporanic acid showed no antibacterial activity under the test conditions used, but formed active *N*-acyl derivatives. A purified sample of 7-aminocephalosporanic acid yielded an *N*-phenylacetyl derivative whose activity against *Staphylococcus aureus* was about 100 times that of cephalosporin C.

3. Treatment of cephalosporin C with dilute acid also yields the nucleus of cephalosporin C_c. The formation of this compound involves hydrolysis of an acetoxy group and subsequent lactonization, as well as removal of the side chain. The same compound is formed on hydrolysis of cephalosporin C_c.

4. Hydrolysis of cephalosporin C_A (pyridine) in dilute acid yields the nucleus of cephalosporin C_A (pyridine). This nucleus is also formed when 7-aminocephalosporanic acid reacts with aqueous pyridine.

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Studies on Vitamin A Esterase

4. THE HYDROLYSIS AND SYNTHESIS OF VITAMIN A ESTERS BY RAT INTESTINAL MUCOSAE*

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Most of the work on vitamin A esterase has been on the hydrolysis of the esters; studies on the esterification are very few. In a preliminary communication High, Bright & Powell (1956) have reported esterification of vitamin A by small intestine and kidneys of rats. Krinsky (1958) has made a rather detailed investigation of esterification of vitamin A by cattle retina. While the present work was in progress, Pollard & Bieri (1960) reported their experiments on the esterification of vitamin A by acetone-dried pancreas of rat and chick.

Since the work of Gray & Cawley (1942) and of Clausen (1943) it has been generally assumed that vitamin A esters undergo hydrolysis and re-esterification during the process of absorption from the small intestine. More recently we have demonstrated that irrespective of whether vitamin A is given orally to rats as the free alcohol, as its acetate or as its palmitate, the esters found in the intestinal muscles, mesenteric lymph, blood and liver are invariably those of higher fatty acids (Mahadevan, Krishnamurthy & Ganguly, 1959), thus supporting the possibility of hydrolysis and re-esterification in the intestine during absorption.

It is demonstrated in this report that the mucosae of the small intestine of rats show hydrolytic and esterifying activities towards the vitamin. Some of the general properties of these activities are described and it is indicated that the esterification of vitamin A is apparently independent of coenzyme A and adenosine triphosphate requirements.

* Part 3. Krishnamurthy, Seshadri Sastry & Ganguly (1958).

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MATERIALS AND METHODS

Materials. Sodium taurocholate and palmitic acid were obtained from British Drug Houses Ltd. The palmitic acid was recrystallized from ethanol before use. Coenzyme A (CoA), *p*-chloromercuribenzoate and the disodium-trihydrate salt of adenosine 5′-triphosphate (ATP) were from Sigma Chemical Co. Tween 20 (polyoxyethylene sorbitan monolaurate) was from the Atlas Powder Co., Wilmington, Delaware, U.S.A., and diethylamine acetarsol (Acetylarsan) from May and Baker Ltd., Bombay, India. Di*iso*propyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP) and diethyl *p*-nitrophenyl phosphate (E–600) were kindly procured for us by Dr S. Y. Thompson of the

National Institute for Research in Dairying, Reading (U.K.) from different sources in England. Sodium barbitone (veronal) was the product of E. Merck. The rest of the materials used have already been described by Ganguly, Krishnamurthy & Mahadevan (1959).

Preparation of the substrates. In our previous studies (Seshadri Sastry, Krishnamurthy & Ganguly, 1957) and in the preliminary experiments of the present study very low concentrations (0·2%, w/v) of Tween 80 (polyoxyethylene sorbitan mono-oleate) were used for dispersing the vitamin A. As will be seen later on, this detergent shows powerful inhibitory effect on the esterifying, but not on the hydrolytic system. Subsequently we found ethanol to be a suitable dispersing medium and all the experiments described here were carried out with ethanol as the dispersing agent (see also Pollard & Bieri, 1960).

The crystalline vitamin A samples (alcohol, acetate or palmitate) were dissolved in light petroleum and were purified by chromatography through alumina columns (Ganguly, Krinsky, Mehl & Deuel, 1952). The ester fractions were evaporated to dryness and taken up in ethanol so as to obtain a final concentration of 500 μg./ml. The vitamin A alcohol was similarly dissolved in ethanol, but here 1 mg. of palmitic acid/ml. was also added.

Preparation and assay of the enzymes. Initially the experiments were carried out with whole homogenates of the intestinal mucosal cells of normal stock rats of this Institute colony. Later on we noticed that feeding with a high-cholesterol diet for about 5 weeks markedly increased the specific activity of the mucosal enzymes of rats (Murthy, Mahadevan & Ganguly, 1960) and it was possible to obtain soluble enzymes in highly active form from acetone-dried powder prepared from the intestinal mucosae of such rats. No appreciable differences in the general properties of the enzymes prepared from these two sources were found and we have therefore used the soluble enzymes for the present investigation.

Young rats of both sexes were kept for 5 weeks on a diet consisting of starch (54%), casein (20%), sucrose (10%), salt mixture (5%), groundnut oil (10%), cholesterol (1%) and adequate supplements of all vitamins, and then were killed. The composition of the diet and the treatment of these animals are described in more detail in Murthy *et al.* (1960). The separation of the contents, mucosae and muscles of the small intestine was carried out as described earlier (Ganguly *et al.* 1959). The mucosae were ground in a pestle and mortar in 10 vol. of acetone at $-15°$. The mixture was centrifuged and the precipitate was washed three times with fresh acetone at $-15°$. Finally the residual acetone was removed under suction. The enzymic activities of the acetone-dried powders, thus prepared, were stable for more than 2 months at 0–5°, but once the enzymes were obtained in water solution they became rather unstable.

For preparation of the soluble enzymes, 1 g. of the acetone-dried powder was kept suspended in 25 ml. of cold water for 1 hr. at 0° and the solid was then removed by centrifuging at 10 000*g* for 15 min. The clear supernatant contained about 30% of the proteins of the original powder and retained about 90% of the activity. It was used as the source of the enzyme for the present study. As far as possible all manipulations were carried out in the cold room at 0–5°.

Unless otherwise stated, the reaction mixtures contained 3 ml. of 0·1 M-veronal buffer (Michaelis, 1931) of the appropriate pH (pH 8·6 for hydrolytic systems and pH 6·6 for esterification), 0·5 ml. of the ethanolic solution of the substrate (prepared as described above) containing 250 μg. of vitamin A alcohol, acetate or the palmitate and 0·5 ml. of the enzyme solution (6 mg. of protein for the hydrolytic reactions and 1·5 mg. of protein for esterification). The hydrolytic system for the palmitate always contained 0·5 ml. of a 10% (w/v) solution of sodium taurocholate, with the exception of the experiments of Tables 1 and 4 and Fig. 6, where the total amounts of the bile salt added are given. The final volume was made up to 5 ml. with water. All incubations were carried out at 37° for 30 min. The reactions were stopped by the addition of 5 ml. of ethanol. The amounts of free and esterified vitamin A formed were then determined according to Seshadri Sastry *et al.* (1957) and were taken as the measures of the enzyme activities.

Proteins were estimated by the biuret method (Robinson & Hogdon, 1940).

RESULTS

Effect of enzyme concentration. As shown in Fig. 1, the hydrolytic activity was proportional to protein concentration up to 10 mg./reaction mixture, and the process of esterification was proportional to protein concentration up to 1·5 mg. The specific activity for the esterification was 24 μg. of vitamin A alcohol esterified/mg. of protein/30 min., whereas for the hydrolysis of the acetate and the palmitate it was 6·7 and 4·0 μg. of alcohol liberated/mg. of protein/30 min. respectively. However, in these experiments bile salt was used with the palmitate, in the absence of which the specific activity

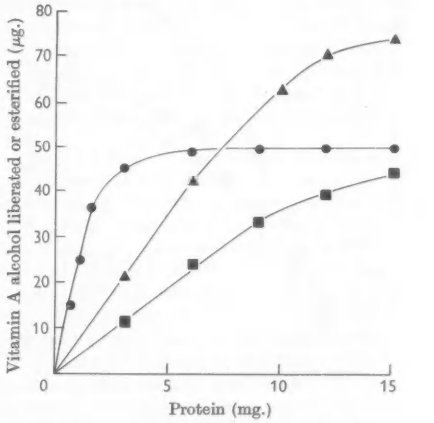

Fig. 1. Effect of enzyme concentration. Conditions employed were the same as those described in the Materials and Methods section. Enzyme extracts containing the given amounts of proteins were added to each reaction mixture. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

for the palmitate hydrolysis was 0·9 μg. of alcohol liberated/mg. of protein/30 min. In subsequent experiments we have used 6 mg. of protein for the hydrolytic studies and 1·5 mg. of protein for the esterification studies.

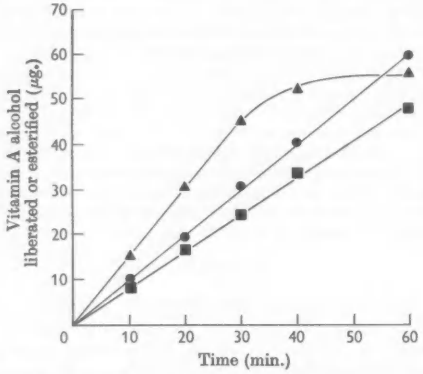

Fig. 2. Time course of the reaction. Conditions were the same as those described in the Materials and Methods section. Samples were withdrawn and analysed at the given time intervals. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

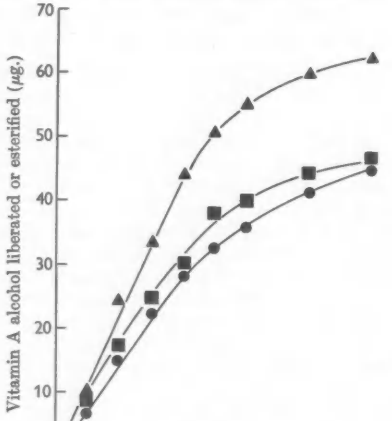

Fig. 3. Effect of substrate concentration. For each reaction a solution of the given amount of the substrate in 0·5 ml. of ethanol was added. Other conditions were the same as those described in the Materials and Methods section. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

Time course of the reactions. Both hydrolysis and synthesis were linear with time up to 30 min. (Fig. 2). The rate of esterification was markedly higher than the rate of hydrolysis of either substrate; the specific activity of the enzyme was almost identical with that found in the experiment described above.

Effect of substrate concentration. The effect of increasing the substrate concentrations on the three activities is shown in Fig. 3.

Effect of pH *and buffers.* The hydrolysis of both the acetate and the palmitate was optimum at pH 8·6, and the esterification had optimum activity at pH 6·6 (Fig. 4). With the rat-liver enzyme (Seshadri Sastry *et al.* 1957) there were two peaks at pH 6·6 and 8·6 for the hydrolysis of the acetate, with optimum activity at pH 8·6. In the present case the smaller peak at pH 6·6 was absent.

Substituting phosphate for veronal buffer did not shift the pH optimum of the esterifying enzyme, although the activity was 30% more with the phosphate buffer. A similar comparison was not made with the hydrolytic systems, as pH 8·6 is outside the range of phosphate buffer. Since the veronal buffer could cover both the pH ranges of 6·6 and 8·6, we chose this particular buffer.

Effect of fatty acid concentration on the esterification. Vitamin A could not be esterified with acetic acid, butyric acid, *iso*valeric acid or hexanoic acid, but it readily formed esters with lauric

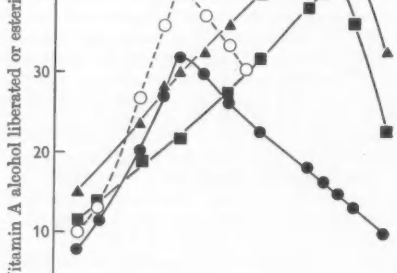

Fig. 4. Effect of pH and buffers. Conditions were the same as those described in the Materials and Methods section. Buffers (0·1 M) of the given pH were used. ▲, Hydrolysis of acetate (in veronal buffer); ■, hydrolysis of palmitate (in veronal buffer); ●, esterification of alcohol (in veronal buffer); ○, esterification of alcohol (in phosphate buffer).

Table 1. *Effect of Tween 20 and sodium taurocholate on hydrolysis and esterification*

Conditions were as described in the Materials and Methods section except that 0·5 ml. of water solutions of Tween or sodium taurocholate containing the corresponding amounts of the detergents were added for each reaction. In the experiments with Tween 20 for palmitate hydrolysis 50 mg. of the bile salt was always present.

	Tween 20 or taurocholate in reaction mixture (mg.)	Vitamin A alcohol (μg.) Liberated		Esterified
		Vitamin A acetate	Vitamin A palmitate	Vitamin A alcohol
Tween 20	0	53·5	26·5	33·0
	2	53·7	27·0	24·5
	5	53·7	26·5	4·3
	10	56·4	11·3	4·3
	25	51·0	8·0	2·8
	50	44·8	8·0	2·8
Taurocholate	0	54·6	5·8	33·0
	10	53·6	15·0	28·5
	25	56·4	18·6	14·3
	50	51·0	26·5	5·7
	100	54·8	29·5	3·6

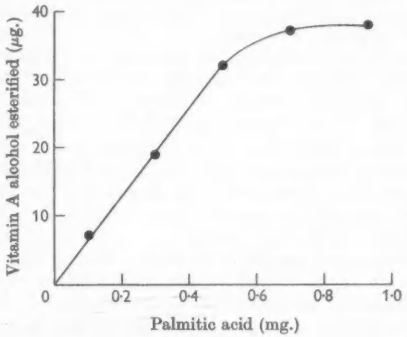

Fig. 5. Effect of fatty acid concentration on esterification. For each reaction 250 μg. of vitamin A alcohol, with the given amount of palmitic acid, in 0·5 ml. of ethanol was added. Other conditions were the same as those described in the Materials and Methods section.

acid, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. The presence or absence of unsaturation in the fatty acid did not appear to make any significant difference in the degree of esterification.

The effect of increasing concentrations of the fatty acid (palmitic acid) on the esterification of vitamin A is shown in Fig. 5. With up to 0·5 mg. of the fatty acid/5 ml. of reaction mixture the esterification was proportional to its concentration and above this concentration there was no further increase in esterification nor was there any inhibition.

Effect of coenzyme A and adenosine triphosphate on esterification. The esterification of vitamin A by the crude soluble enzymes was independent of CoA and ATP. Even with enzyme preparations purified 20- to 30-fold and dialysed for 24 hr. against cold distilled water there was no requirement for CoA or ATP. Similar results were obtained with cattle retina (Krinsky, 1958) and with chick pancreas (Pollard & Bieri, 1960). Recent work on the biosynthesis of triglycerides and phospholipids (Weiss, Kennedy & Kiyasu, 1960) and on acetylation of choline (Berman, Wilson & Nachmansohn, 1953) has shown that in some esterification reactions the fatty acids must be activated to the acyl-CoA derivatives by CoA and ATP; nevertheless, all esterification reactions do not seem to be energy-dependent. Thus not only the esterification of vitamin A, but the esterification of cholesterol by hog pancreas (Hernandez & Chaikoff, 1957) or by rat-intestinal mucosae, is independent of CoA and ATP (S. K. Murthy, S. Mahadevan & J. Ganguly, unpublished work).

Effect of Tween 20 and sodium taurocholate. In previous studies with the hydrolytic enzymes Tween dispersions of vitamin A were consistently used (McGugan & Laughland, 1952; Ganguly & Deuel, 1953; Ganguly, 1954; Seshadri Sastry *et al.* 1957). However, Tween compounds appear to act as inhibitors for the esterification of vitamin A (Krinsky, 1958). The effect of Tween 20 on the systems from the intestinal mucosae was therefore investigated. The detergent had little or no effect on the hydrolysis of the acetate and the palmitate up to a concentration of 5 mg./reaction mixture, but at higher concentrations the palmitate hydrolysis was inhibited to about 70% (Table 1). At the same time it had a pronounced inhibitory action on the esterifying reaction. Thus even at a concentration of 5 mg./reaction mixture the inhibition was about 85%. In separate experiments without the tauro-

cholate, Tween 20 up to a concentration of 5 mg./reaction mixture had no appreciable effect on the hydrolysis of the palmitate. Our preliminary results with Tween 80 (polyoxyethylene sorbitan monooleate) indicated that this compound was an even more potent inhibitor of the esterification. These results thus confirm the observations of Krinsky (1958). Cleland & Kennedy (1960) have reported that Tween 20 inhibits the biosynthesis of psychosine.

In contrast, as further shown in Table 1, the bile salt had a marked activating effect on the hydrolysis of the palmitate, without exerting similar action on the acetate hydrolysis. Thus the acetate was hydrolysed equally well irrespective of whether the bile salt was absent or present even at a concentration of 50 mg., whereas the palmitate hydrolysis had increased 4·4-fold with a change in the concentration of the detergent from 0 to 50 mg. The hydrolysis of the palmitate was only about 5 μg. of vitamin A alcohol liberated/30 min./reaction mixture without the bile salt. This is obviously too low for studies of reasonable accuracy. We have therefore used 50 mg. of bile salt/reaction mixture for the hydrolytic system for the palmitate.

The esterification, however, was progressively inhibited with increasing concentrations of the bile salt. This is quite different from the behaviour of cholesterol, where both hydrolysis and esterification by the same mucosal enzymes require the presence of the bile salt (Swell & Treadwell, 1955; S. K. Murthy, S. Mahadevan & J. Ganguly, unpublished work).

Effect of inhibitors

Metal ions, alkaloids and arsenicals. The effects of the various compounds on the activities are summarized in Table 2. Most of the compounds tested did not have any appreciable effect on the three systems at a final concentration of 0·1 mM. However, at 1 mM concentration some interesting differences in the three systems were evident. Thus calcium chloride had considerable activating effect on the hydrolysis of the palmitate and not of the acetate, but at the same time it had an inhibitory action on the esterification process. It is possible that this may be due to the formation of insoluble calcium salts of the free palmitic acid. With the other compounds, there was variation in the degree of inhibition of the three systems. Three compounds, however, sodium fluoride, sodium arsenite and diethylamine acetarsol, had no effect on esterification but produced considerable inhibition of the hydrolysis of both the acetate and the palmitate. Many of the compounds listed in Table 2 displayed similar effects on the hydrolysis of vitamin A acetate by rat-liver vitamin A esterase (Seshadri Sastry *et al.* 1957).

p-Chloromercuribenzoate. This compound had no effect on the three systems. P. Seshadri Sastry & J. Ganguly (unpublished work) have observed that the hydrolysis of vitamin A acetate by rat- and chicken-liver enzymes is inhibited by *p*-chloromercuribenzoate and, according to Krinsky (1958), the esterifying enzyme of cattle retina is also inhibited by this reagent, but Pollard & Bieri (1960) do not consider the chick-pancreas esterifying enzyme to be a sulphydryl one. It is possible that these discrepancies could be due to the fact that not only are they from different tissues, they belong to different species also.

Organophosphorus compounds. Several organophosphorus compounds have been used by other workers for differentiating the esterases of animal tissues (Myers, Schotte & Mendel, 1955). By the procedure described by Aldridge (1954), the effects of three of these compounds, DFP, TEPP and E–600, on the three systems were studied (Table 3).

Table 2. *Effect of metal ions, alkaloids and arsenicals on the hydrolytic and esterifying systems*

Except for the substrate, all other components described in the Materials and Methods section were preincubated with 0·5 ml. of aqueous solution of the given compound for 15 min. at 37°, after which the substrate was added and incubation continued for another 30 min. at the same temperature.

	Activity retained (%)					
	Vitamin A acetate		Vitamin A palmitate		Vitamin A alcohol	
Compound	1 mM	0·1 mM	1 mM	0·1 mM	1 mM	0·1 mM
$HgCl_2$	55·5	88·0	47·5	94·0	65·0	97·8
$MgSO_4,7H_2O$	88·6	97·0	100·0	100·0	85·4	86·4
$CaCl_2$ (anhydrous)	106·0	99·8	162·0	112·0	66·0	84·0
$CuSO_4,5H_2O$	18·4	87·0	65·0	96·4	30·0	92·1
$ZnCl_2$	11·1	69·6	31·0	93·0	18·1	91·2
NaF	79·6	96·0	67·0	95·6	96·3	97·4
NaCN	101·0	99·8	70·1	98·6	100·0	100·0
Na_3AsO_3	55·0	87·0	60·0	96·0	100·0	100·0
Diethylamine acetarsol	46·0	77·7	60·8	95·2	96·6	100·0
Quinine–HCl	60·0	92·8	55·0	98·8	30·0	96·3

Table 3. *Effect of organophosphorus compounds on the hydrolytic and esterifying systems*

Conditions and procedures were the same as described for Table 2.

	Inhibition (%)					
Final concn. of inhibitor (μM) ...	100	10	1	0·1	0·01	0·001
Di*iso*propyl phosphorofluoridate						
Vitamin A acetate	100·0	100·0	100·0	100·0	53·6	5·2
Vitamin A palmitate	74·6	50·0	33·6	2·8	0	0
Vitamin A alcohol	4·8	3·5	0	0	0	0
Diethyl *p*-nitrophenyl phosphate						
Vitamin A acetate	100·0	100·0	100·0	89·0	43·0	14·0
Vitamin A palmitate	72·8	35·0	8·0	0	0	0
Vitamin A alcohol	4·8	4·0	0	0	0	0
Tetraethyl pyrophosphate						
Vitamin A acetate	100·0	100·0	100·0	88·5	26·4	6·6
Vitamin A palmitate	0	0	0	0	0	0
Vitamin A alcohol	0	0	0	0	0	0

Table 4. *Effect of sodium taurocholate on the hydrolytic and esterifying systems in the presence of* 0·1 mM-*di*isopropyl *phosphorofluoridate*

For the hydrolysis of the palmitate the enzyme was preincubated in 0·1 M-veronal buffer, pH 8·6, with 0·1 mM-DFP as described in Table 3. The taurocholate and the substrate were then added and the incubation was continued at 37° for 30 min. The esterification was at pH 6·6 (veronal buffer, 0·1 M) after preincubation of the enzyme with 0·1 mM-DFP as described in Table 3. The rest of the procedure was the same as for hydrolysis.

	Vitamin A alcohol (μg.)	
	Liberated	Esterified
Additions	Vitamin A palmitate	Vitamin A alcohol
Without taurocholate	5·8	35·0
With taurocholate (50 mg.)	35·0	7·0
With taurocholate (50 mg.) + 0·1 mM-DFP	8·7	7·0
With 0·1 mM-DFP	1·2	35·0

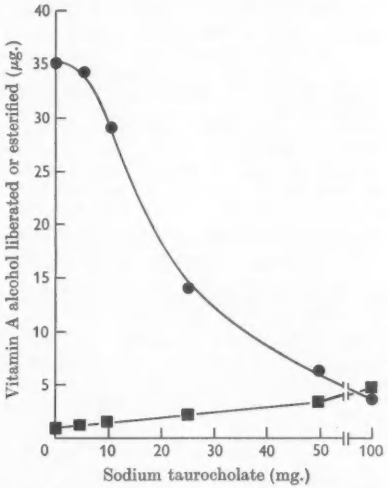

Fig. 6. Effect of sodium taurocholate in the presence of DFP. Reactions were carried out in a total volume of 5 ml. containing a constant amount of DFP (0·1 mM) and the given amounts of the bile salt, as described in the Materials and Methods section. ■, Hydrolysis of palmitate; ●, esterification of alcohol.

With all three of them there was total inhibition of the hydrolysis of the acetate at inhibitor concentrations of 1 μM–0·1 mM. Two of the three inhibitors (DFP and E–600) had considerable effect on the hydrolysis of the palmitate, though it was not as pronounced as with the acetate. The third inhibitor (TEPP) does not appear to have any effect on the hydrolysis of the palmitate.

The esterification reaction, on the other hand, was completely unaffected by all of these compounds. It would thus appear logical to conceive that the esterification of vitamin A by the mucosal enzymes is not a direct reversal of the hydrolytic process and this is further supported by the following experiments with DFP and taurocholate.

*Sodium taurocholate in the presence of di*isopropyl *phosphorofluoridate*. There was little hydrolysis of the palmitate without the bile salt, but the esterification was inhibited by this agent. DFP alone inhibited the hydrolysis, but not the esterification, whereas these two substances added together inhibited both the hydrolytic and esterification reactions (Table 4). In Fig. 6 it is further demonstrated that in the presence of a constant amount of DFP increasing concentrations of the bile salt produced progressive inhibition of the esterification without concomitant increase in hydrolysis.

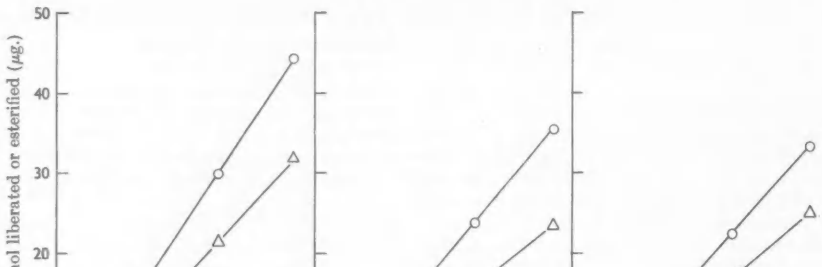

Fig. 7. Intracellular distribution of the hydrolytic and synthetic activities of intestinal mucosae of normal rats. For each reaction, cell fractions equivalent to 100 mg. of the original mucosal tissue were used. Other conditions were similar to those described in the Materials and Methods section. (*a*) Hydrolysis of acetate; (*b*) hydrolysis of palmitate; (*c*) esterification of alcohol. ○, Whole homogenate; ●, nuclear fraction; ×, mitochondrial fraction; △, microsomal fraction; □, supernatant fraction.

Intracellular distribution of the hydrolytic and esterifying activities of the mucosae

The mucosae of the small intestine of normal stock male rats were collected, homogenized in 0·25 M-sucrose and fractionated by differential centrifuging into the nuclear, mitochondrial, microsomal and supernatant fractions as described by Ganguly *et al.* (1959). Fig. 7 shows that these activities are mostly localized in the microsomal fraction of the homogenate. Earlier workers found the hydrolytic and esterifying systems of the cells of other tissues also to be associated with the particulate materials of the cell homogenate. Thus the hydrolytic enzyme is in the microsomal fraction in rat liver (Ganguly & Deuel, 1953) and in the nuclear and microsomal fractions in chicken liver (Krishnamurthy, Seshadri Sastry & Ganguly, 1958); the esterifying enzyme in cattle retina is sedimentable by centrifuging at 35000*g* (Krinsky, 1958).

Other properties of the mucosal enzymes. Prolonged dialysis of the mucosal-cell homogenates for 24 hr. against frequently changed cold distilled water did not alter the activities to any appreciable extent. The homogenate enzymes were stable for at least 30 days when stored at 4°, but the soluble enzymes, as prepared from the acetone-dried mucosae, lost 40% of their activities at the same temperature in 1 day.

Distribution of the activities in normal rats. The activities were present in measurable amounts in the contents, mucosae and muscles of the small intestine, as well as in the pancreas of the normal stock rats (Table 5). The specific activities of the enzymes of intestinal contents were the highest. The kidneys did not show any appreciable activity

Table 5. *Tissue distribution of the activities in the normal stock rat*

Conditions were the same as in the Materials and Methods section except that 5–20 mg. of protein was used for each reaction.

	Vitamin A alcohol (μg./mg. of protein/30 min.)		
	Liberated		Esterified
	With 50 mg. of bile salt	Without bile salt	Without bile salt
	Vitamin A palmitate		Vitamin A alcohol
Pancreas	13·0	6·4	7·6
Small intestine			
Contents	40·0	27·0	55·5
Mucosae	2·3	Nil	2·6
Muscle	9·2	Nil	5·3
Plasma	Nil	Nil	0·1
Liver	0·13	Nil	Nil
Kidneys	0·12	Nil	0·2
Spleen	0·08	Nil	0·25

with respect to esterification and this seems to agree with the findings of Pollard & Bieri (1960), but not with those of High *et al.* (1956).

DISCUSSION

The work of Gray & Cawley (1942) had implied the presence of both hydrolytic and synthetic activities for vitamin A esters in the small intestine and our studies have demonstrated that these activities are present not only in the lumen, but also in the muscles and mucosae of the small intestine of rats. Rapid hydrolysis of vitamin A esters and esterification of the free vitamin A can be demonstrated with the mucosal-cell homogenates, as well as with the soluble enzymes prepared from the acetone-dried mucosae.

Optimum rates of hydrolysis took place at pH 8·6, whereas the esterification was optimum at pH 6·6. Sodium arsenite, sodium fluoride and diethylamine acetarsol were able to inhibit partially the hydrolysis, but not the esterification. On the other hand, Tween 20 even at 0·1 % concentration exerted a pronounced inhibitory action on the esterification, but at the same time had no effect on the hydrolysis. Finally the organophosphorus compounds were very powerful inhibitors of the hydrolytic process only. It would thus appear probable that though both activities are recovered from the microsomal fraction of the cell homogenate of the mucosae, the hydrolysis and esterification are not brought about by the reversal of one reaction.

Instances, however, are known where hydrolysis and esterification are not strictly reversible, e.g. the enzymic formation of choline esters (Berman *et al.* 1953) or of triglycerides (Weiss *et al.* 1960). But in such cases the fatty acids pass through a state of activation in the form of acyl-CoA derivatives at the expense of ATP. However, no CoA or ATP seems to be necessary for the esterification of vitamin A or of cholesterol. The exact mechanisms of such esterification processes thus remain obscure.

The fatty acid-specificity experiments have indicated that the esterification of vitamin A by the mucosal enzymes takes place with fatty acids containing more than 10 carbon atoms without any preference for chain length or unsaturation of the acid. Gray & Cawley (1942) have suggested that in rat liver vitamin A is probably stored as its palmitate. More recent work has shown that after feeding of vitamin A acetate the vitamin appears in the muscles of the small intestine as its higher ester (Mahadevan *et al.* 1959). This suggests that the vitamin A esters undergo hydrolysis and are then esterified again inside the mucosal cells after absorption. The most common fatty acids in the animal body and in the normal diet, and available for esterification of vitamin A at the site of absorption, are stearic acid, palmitic acid, oleic acid and linoleic acid. This would therefore explain why only the higher fatty acid esters are found in the animal body (Kaiser & Kagan, 1956).

Poor or no absorption of vitamin A (presumably the natural esters) in biliary diseases is a well-recognized fact; oral administration of extra bile along with the vitamin improves its absorption in such cases (Clausen, 1943). We have demonstrated here that the hydrolysis of the higher esters of the vitamin is rather poor without the bile salt and is greatly facilitated by its presence. These results would thus explain the role of the bile in improving the absorption of vitamin A in biliary diseases.

SUMMARY

1. The hydrolytic and synthetic activities for vitamin A esters are present in the contents, mucosae and muscles of the small intestine, as well as in the pancreas of rats, with the luminar enzymes showing highest specific activities.

2. Both the activities are localized in the microsomal fraction of the mucosal-cell homogenate.

3. The activities of the mucosae can readily be brought into solution by extracting the acetone-dried mucosae with water.

4. Hydrolysis of vitamin A acetate by the soluble enzymes did not require sodium taurocholate; that of the palmitate was very poor in the absence of the bile salt and was greatly activated by it.

5. The hydrolytic and esterifying activities were optimum at pH 8·6 and 6·6 respectively. The hydrolytic activity was preferentially inhibited by sodium fluoride, sodium arsenite, diethylamine acetarsol, di*iso*propyl phosphorofluoridate and diethyl *p*-nitrophenyl phosphate. Tween 20, sodium taurocholate and calcium chloride inhibited esterification alone.

6. No coenzyme A or adenosine triphosphate appeared to be necessary for the esterification of vitamin A.

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The Occurrence of Free D-Serine in the Earthworm

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The occurrence of D-serine in lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate) and serine ethanolamine phosphodiester, isolated from the earthworm, has been reported from this laboratory (Beatty, Magrath & Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960; Beatty, Ennor, Rosenberg & Magrath, 1961).

This paper describes the isolation of DL-serine from earthworm tissues and provides conclusive proof that D-serine is present in the tissues immediately before extraction and that it could not arise as a result of racemization of L-serine during the isolation procedure.

A preliminary report of this work has appeared (Rosenberg & Ennor, 1960).

MATERIALS AND METHODS

All reagents were A.R. grade. Ethanol used for chromatography and other purposes was purified by refluxing over KOH and aluminium powder and then by fractional distillation. D-, L- and DL-Serine were purchased from California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A. L-[3-^{14}C]serine was obtained from Dr R. L. Blakley of this Department and was prepared from glycine and [^{14}C]formaldehyde with serine transhydroxymethylase (McDougall & Blakley, 1961). The earthworms used in this work were as described by Rosenberg & Ennor (1959).

Isolation of serine. The experimental procedures used for the isolation of serine from the tissue have been described in detail (Ennor *et al.* 1960), and the various steps will be referred to in general terms only. Such modifications as have been introduced will be described in the text.

D-*Amino acid oxidase.* This was prepared according to Negelein & Brömel (1939) and was taken to stage III [$(NH_4)_2SO_4$ precipitation]. The precipitate containing the enzyme was stored at 0° as a suspension in 50% saturated $(NH_4)_2SO_4$. Portions of this suspension were centrifuged as required and the precipitate was resuspended in 0·01 M-pyrophosphate buffer, pH 8·1, and dialysed against 100 vol. of the same buffer, with two changes, for 24 hr. The dialysed solution was cleared by centrifuging and its volume adjusted with water so that each millilitre of the solution corresponded to 1 g. of the acetone-dried kidney powder used in the preparation. The solution was tested for the presence of catalase according to Herbert (1955) and it was found that 0·1 ml. of the solution decomposed 100 μmoles of H_2O_2 in 30 sec. The solution was kept frozen at −10° and retained activity for at least 3 months. Assays with this enzyme were carried out in Warburg manometers at 37° in air. The vessels contained 2·0 ml. of 0·1 M-sodium pyrophosphate buffer, pH 8·3, the amino acid tested and 0·3 ml. of the enzyme solution, in a total volume of 3 ml.

Ascending paper chromatography. This was carried out on Whatman no. 3 papers and the following solvent systems were employed: (I) ethanol–formic acid–water (70:10:20, by vol.); (II) water-saturated phenol; (III) butan-2-one–methylCellosolve (2-methoxyethanol)–acetic acid–water (40:15:6:24). Amino acids and related compounds were detected by dipping the dried papers into 0·2% solution of ninhydrin in acetone, followed by heating for 10 min. at 80°.

Table 1. *Influence of alkali on the racemization of* L-*serine as tested with* D-*amino acid oxidase*

Full experimental details of the method employed and of the treatment with alkali are given in the text.

Substrate	D-Serine	L-Serine	L-Serine	L-Serine
Amount present (μmoles)	10·0	238	238	238
Treatment	Nil	Nil	Aq. 2·5 N-NH_3 (48 hr.)	Satd. $Ba(OH)_2$ (48 hr.)
Uptake of O_2 (μg.atoms/3 hr.)	10·0	0	0·9	0

Radioactivity determinations. These were carried out as described by Rossiter, Gaffney, Rosenberg & Ennor (1960). Samples of crystalline serine (1–2 mg.) were weighed directly into the counting vessels. Samples (20 μl.) of aqueous fractions emerging from columns were dried in the counting vessel at 110° but, whenever the solutions contained mineral acid, excess of 2 N-ammonia was added before drying to avoid discoloration.

RESULTS

Optical stability of L-*serine*

Daft & Coghill (1931) isolated DL-serine from acid hydrolysates of protein after treatment with excess of $Ba(OH)_2$ and assumed that racemization took place whilst the solution was alkaline. Because of these findings it seemed desirable to ensure that the D-serine isolated from perchloric acid extracts of earthworms (Rosenberg & Ennor, 1960) had not arisen as a result of exposure to alkali. The procedure employed in these experiments was similar to that described for the isolation of lombricine and serine ethanolamine phosphodiester (Ennor *et al.* 1960). This involved a desalting step, which consisted in the passage of the neutralized and filtered extract through Zeo-Karb 225 ion-exchange resin (H^+ form) followed by elution of the adsorbed material with aq. 2·5 N-NH_3 soln. This eluate, which was salt-free, was then taken to near-dryness under reduced pressure in a rotary evaporator at 40°, at which stage it was NH_3-free and pH 7–8. The subsequent fractionation and purification did not involve any treatment at pH greater than 7.

The present investigation into the effects of alkaline solutions on the optical homogeneity of L-serine has been carried out with both aq. 2·5 N-NH_3 soln. and aq. saturated $Ba(OH)_2$. A quantity of L-serine (0·5 ml. of 5 %, w/v, soln.) was placed in each of two flasks: to one of these was added 2 ml. of aq. 2·5 N-NH_3 soln. and to the other 2 ml. of saturated $Ba(OH)_2$ soln. Both flasks were allowed to stand at room temperature (about 22°) for 48 hr., when the NH_3 was removed by evaporation as before. The $Ba(OH)_2$ soln. was passed through a column (6 cm. × 1·2 cm.) of Dowex-50 ion-exchange resin (NH_4^+ form), which was then washed with water. The combined effluent and washings contained serine in addition to the ammonia displaced by the Ba^{2+} ion. The ammonia was removed by evaporation and both dry residues were dissolved in water (0·7 ml.) and quantitatively transferred to the main chamber of Warburg manometric flasks. The ability of the serine present in these flasks to act as a substrate for D-amino acid oxidase was then compared with that of samples of authentic L- and D-serine. The results (Table 1) show that no detectable racemization occurred with the baryta treatment but that some racemization did occur in the presence of ammonia. This, however, was small and on the basis of the uptake of O_2 amounted to only 0·4 % of the serine present. This degree of racemization was not considered serious, particularly in view of the fact that the earlier isolation procedure (Rosenberg & Ennor, 1960) involved contact with NH_3 for no longer than 30 min. Moreover, the comparative yields of DL- and L-serine reported would have necessitated an inversion to the extent of at least 25 %. Such a change in the optical purity would seem most unlikely in view of the above-mentioned results but it was nevertheless decided to carry out an additional control experiment. The behaviour of an authentic sample of L-serine (50 mg. in 200 ml. of 1·5 N-$HClO_4$) has been checked through the entire isolation procedure and tested enzymically for the presence of D-serine as before. The results (Table 2) show that no detectable degree of racemization of serine occurred. This and the preceding experiments provide conclusive proof of the safety of the extraction and fractionation procedures in so far as the retention of optical homogeneity is concerned. But some doubt may remain when such procedures are applied to earthworm tissue; it seemed possible that the latter might contain some unrecognized factor(s) which would catalyse racemization in acid or alkaline solution. This possibility has been examined by employing a variant of the isolation technique which did not involve the use of an alkaline medium at any stage and which employed

Table 2. *Action of* D-*amino acid oxidase on a serine-containing fraction isolated from rabbit muscle and serine obtained after passage of* L-*serine through the isolation procedure.*

Substrate ...	D-Serine	L-Serine (authentic)	L-Serine reisolated
Amount present (μmoles)	10·0	238	200
Uptake of O_2 (μg.atoms/2 hr.)	9·6	0	0·3

an internal check on racemization through the addition of L-[3-^{14}C]-serine.

Approximately 5 kg. of frozen earthworms was extracted in a Waring Blendor with 1·5 vol. of 1·5N-$HClO_4$. Labelled L-serine (20 μmoles) was added to the resultant suspension, which was then treated as described by Ennor *et al.* (1960), except that the NH_3-elution stage was avoided as follows. After adsorption on to a cation-exchange resin (Dowex 50, H^+ form; mesh 50–100), the material was eluted with 0·2N-$HClO_4$ instead of NH_3. The eluate was collected in 25 ml. fractions and those containing serine were located by radioactivity determinations. These were pooled, adjusted to pH 7 by the addition of 10N-KOH at 0° and the precipitated $KClO_4$ was removed by filtration. The filtrate was transferred to a column (3 cm. × 55 cm.) of Amberlite CG-120 (H^+ form) and the adsorbed material eluted by gradient elution with $HClO_4$ as described by Ennor *et al.* (1960).

Those fractions containing serine were identified by measurement of radioactivity, combined and the perchloric acid was removed as described above. The resultant solution of serine was evaporated under reduced pressure to about 5 ml. and further purification of the serine was achieved by repeated passage through columns of Dowex 50 (NH_4^+ form) as described by Ennor *et al.* (1960). After each passage those fractions containing serine were located as described above and then examined by paper chromatography in solvent II. In this way those fractions containing uncontaminated serine were located. They were then combined and stored at −10°. The fractions containing serine contaminated with other amino acids were combined and again passed through Dowex 50 (NH_4^+ form). After three such passages the bulk of the serine was free from contamination by other amino acids and the combined solutions of uncontaminated serine were evaporated under reduced pressure at 30° to about 7 ml. Ethanol (about 10 ml.) was added to this residue and crystallization commenced immediately. The mixture was allowed to stand overnight at room temperature and filtered to yield crystalline plates (crop I), which corresponded to those referred to in the preliminary communication (Rosenberg & Ennor, 1960) and which were found to contain mainly DL-serine. This was repeatedly recrystallized from aq. ethanol. The filtrate from which crop I was obtained was treated with a further 20 ml. of ethanol and kept at 0° for 24 hr. Long fine needles of L-serine (crop II) were recovered by filtration and the mother liquor was discarded. The crystals were repeatedly recrystallized from aq. ethanol. (Found: crop I, C, 33·97; H, 6·68; N, 13·19. Crop II, C, 33·95; H, 6·69; N, 13·08. $C_3H_7O_3N$ requires C, 34·28; H, 6·72; N, 13·33 %). Comparison of the yields (Table 3) of DL- and L-serine (crops I and II respectively) indicates that about 20 % of the serine isolated was in the D-form. The results of the radioactivity determinations (Table 3) show that the specific radioactivity of the DL-serine was approximately one-half of that of the L-serine. The small excess of radioactivity of the serine racemate over that demanded by theory could represent a maximum of 5 % conversion of L-serine into the D-enantiomorph. In order to obtain a more precise estimate of any possible conversion of L-serine into the D-isomer 40 mg. of the racemate was dissolved in 2 ml. of water and 70 mg. of authentic D-serine was added. The solution was warmed and mixed with 3 ml. of ethanol and allowed to stand for 18 hr. at room temperature. The crystals which separated were found to have a specific radioactivity of 7700; they were assumed to be predominantly racemate (with D-serine contamination) and were discarded. D-Serine was crystallized from the supernatant after the addition of 20 ml. of ethanol. The crystals were collected and dried, and the radioactivity was assayed in a small sample. The remainder was dissolved in water (2 ml.), ethanol (1·5 vol.) was added and the mixture allowed to stand overnight at room temperature to permit crystallization of DL-serine. This was removed by filtration and discarded, and additional ethanol was added as before to permit crystallization of the D-isomer. This was recovered and the specific radioactivity again measured on a small sample. This second recrystallization resulted in a slight fall in specific radioactivity (Table 4). The remainder of the D-serine was again dissolved in water and about 1 mg. of authentic L-serine added. DL- and D-Serine were crystallized from this solution by a repetition of the procedure described and the procedure was again repeated after a further addition of L-serine. The third and fourth recrystallizations resulted in a sharp fall in the specific radioactivity (Table 4) and indicates that the excess counts found in the racemate were not due to the presence of D-[^{14}C]-serine.

Stability of lombricine in worm homogenates

Some attention has been given to the question of whether the D-serine found arises from the breakdown of lombricine or D-serine ethanolamine phos-

Table 3. *Specific radioactivities and physical characteristics of serine isolated from earthworm extracts to which* L-[^{14}C]*-serine had been added*

	DL-Serine (crop I)	L-Serine (crop II)
Yield (mg.)	105	140
M.p. (decomp.)	244°	222°–223°
$[\alpha]_D^{23}$ in water	0·0 (*c*, 4·0)	−7·2 (*c*, 5·97)
Specific radioactivity (counts/min./mg.)	8,790	16,970

phodiester or both, particularly as Robin (1954) has reported the ability of the insoluble particles of worm homogenates to degrade lombricine to guanidinoethyl phosphate and serine. A homogenate was prepared by homogenizing one live worm (*Megascolides cameroni*) (7 g.) in 5 ml. of water. This crude homogenate was made to 15 ml. by the addition of water and was tested for its ability to degrade lombricine and D-serine ethanolamine phosphodiester. The reaction mixtures tested contained 1·0 ml. of homogenate and 1·0 ml. of either phosphate buffer (0·1 M, pH 7·0) or pyrophosphate buffer (0·1 M, pH 8·3) and 5 mg. of test substance in a total volume of 5·0 ml. The tubes with appropriate controls were incubated for 4 hr., when 2·0 ml. of 3·5 N-$HClO_4$ was added. The resultant mixtures were centrifuged and the supernatant fluid was brought to pH 7·0 by the addition of 2 N-KOH at 0°. The precipitates of $KClO_4$ were removed by filtration and the filtrates evaporated to dryness *in vacuo*. The residues were dissolved in 1·0 ml. of cold water and filtered to remove insoluble $KClO_4$. Portions of the resultant clear solutions were spotted on to paper together with markers of authentic samples of lombricine, 2-guanidinoethyl phosphate, 2-guanidinoethanol and serine, and the chromatograms were developed in solvents II and III and sprayed with ninhydrin and α-naphthol–diacetyl reagents respectively. In addition, two-dimensional chromatograms were developed in solvent systems I and II and sprayed with ninhydrin. The unidimensional chromatograms showed the presence of trace amounts of 2-guanidinoethyl phosphate in all the incubated mixtures together with traces of an unidentified α-naphthol–diacetyl-positive compound with R_F 0·9, which was greater than that of 2-guanidinoethanol, the next fastest-running marker compound. Lombricine was present in all reaction mixtures and the intensity of the lombricine spots showed no lessening with either increasing incubation time or with pH. The two-dimensional chromatograms were confirmatory in the sense that there was no evidence of any disappearance of lombricine, or D-serine ethanolamine phosphodiester, or of any appearance of the expected degradation products.

Table 4. *Effect of repeated recrystallization on the specific radioactivity of* D-*serine*

D-Serine was isolated from a mixture of 40 mg. of DL-[^{14}C]serine (cf. Table 3) and 70 mg. of authentic D-serine.

Compound		Specific radioactivity (counts/min./mg.)
Original DL-serine		8790
D-Serine	first crystallization	288
	second crystallization	238
	third crystallization	75*
	fourth crystallization	37*

* In these recrystallizations about 1 mg. of unlabelled L-serine was added as carrier.

DISCUSSION

The unique nature of the finding (Rosenberg & Ennor, 1960) of a D-amino acid in the free amino acid pool of animal tissue makes it desirable that the evidence for its presence should be accompanied by unequivocal proof that the 'unnatural' isomer could not have arisen as a result of racemization possibly induced by the isolation procedure.

The subject of the present investigation was the earthworm, which has already been shown to contain lombricine and D-serine ethanolamine phosphodiester, in both of which substances the serine component is present as the D-isomer (Beatty *et al.* 1959; Ennor *et al.* 1960; Beatty *et al.* 1961). In addition therefore to the possibility that the D-serine may have arisen as a result of racemization, there is a possibility that it may have arisen as a result of a bio-degradation of lombricine or D-serine ethanolamine phosphodiester or both. Both of these possibilities have been investigated in the present work.

The effect of alkali in producing racemization of serine appears to have met with general acceptance (cf. Neuberger, 1948; Desneulle, 1953). This is based on the report of Daft & Coghill (1931), who claimed without proof that L-serine in protein hydrolysates was racemized on treatment with barium hydroxide. Crawhall & Elliott (1951), using optical rotation as a criterion, failed to detect any change in the optical purity of serine in N-sodium hydroxide for 22 hr. at room temperature. The present results confirm and amplify those of Crawhall & Elliott (1951) in that they demonstrate the optical stability of serine to alkali by using an enzymic method which affords a greater degree of sensitivity than the measurement of optical rotation. Barium hydroxide was shown to be without effect on the optical purity of L-serine. The exposure of 238 μmoles of L-serine to aqueous 2·5 N-ammonia solution at room temperature for 48 hr. did lead to a detectable uptake of oxygen when tested with D-amino acid oxidase. If it be assumed that this was due to D-serine the amount present could not have been more than 1 μmole and thus would represent less than 0·5 % conversion of L-serine into the D-enantiomorph. Racemization as a result of exposure of L-serine to alkaline solvents can therefore clearly be excluded as an explanation for the large amount (25 %) of the D-isomer found in association with the L-enantiomorph.

When ammoniacal solvents were replaced by acidic ones no detectable degree of racemization occurred and, under these conditions, the occur-

rence of D-serine was again demonstrated. The further possibility that racemization may be 'catalysed' by some compound(s) in the earthworm extract has also been investigated. In these experiments L-[^{14}C]-serine was added to perchloric acid suspensions of earthworm tissue and use was made of the difference in solubilities of D- and L-serine and the racemic mixture (cf. Minthorne & Koeppe, 1960). Theoretically the latter would be expected to possess precisely one-half of the specific radioactivity of the L-serine present in the extract and experimentally this has been found nearly to be the case. In actual fact the DL-serine contained 8790 counts/min./mg. as compared with the theoretical expectation of 8490 counts/min./mg. That this difference was probably due to experimental error and not to racemization was indicated by the progressive fall in radioactivity of the isolated D-serine after successive recrystallizations. The nature of this fall and the relatively small amount of radioactivity which remained after the fourth recrystallization strongly suggest that a zero level of radioactivity would have been reached had further recrystallizations been possible. These, however, were precluded because of the small amount of material which remained at this stage. In view of these results it is concluded that the D-serine isolated could not have originated in a racemization induced by acid, alkaline or resin treatment of the earthworm extracts.

There remains the final possibility that the D-serine may have arisen as a result of a post-mortem enzymic breakdown of lombricine or D-serine ethanolamine phosphodiester or both. This possibility cannot be dismissed from consideration with the same degree of certainty as can those already discussed, for, although the present experiments lend no support, the fact remains that complete breakdown of lombricine was achieved in Robin's (1954) experiments at pH 8·0 in 3 hr. Robin (1954) assumed that the breakdown observed was enzymic in nature, but no evidence was adduced to support this assumption and in this connexion it is noteworthy that lombricine (and serine ethanolamine phosphodiester) is extremely labile at room temperature in alkaline solution in the presence of traces of Cu^{2+} ion (Beatty & Magrath, 1960).

The presence of D-amino acids has been recognized in some micro-organisms and in the products of mould and bacterial metabolism, but in none of these has a free D-amino acid been found. So far as we are aware the presence of a free D-amino acid has been described only once before in Nature, when D-alanine was detected in insect (*Oncopeltus fasciatus*) haemolymph by Auclair & Patton (1950).

The present results establish beyond reasonable doubt that D-serine is a component part of the free amino acid pool in the earthworm, but whether this arises from a catabolic reaction involving lombricine (and D-serine ethanolamine phosphodiester), a synthesis of this isomer *de novo*, a racemization of L-serine or some other process, cannot at present be determined. Experiments designed to determine its origin and its mode of incorporation into D-serine ethanolamine phosphodiester are in progress.

SUMMARY

1. D-Serine has been isolated as the racemate from perchloric acid extracts of earthworms.

2. It has been shown that the D-serine isolated is unlikely to have arisen as a result of racemization due to the isolation procedures or of any enzymic breakdown of lombricine or of D-serine ethanolamine phosphodiester.

3. D-Serine is presumed to be a part of the free amino acid pool in the earthworm.

4. Experimental evidence has been adduced to show that, contrary to accepted beliefs, the optical homogeneity of serine is unaffected by exposure to alkaline solvents.

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Estimation of Calcium and Magnesium in Blood Serum by the Cathode-Ray Polarograph

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In our hands precipitation (Clark & Collip, 1925) and visual complexometric (Buckley, Gibson & Bortolotti, 1951) methods of determining calcium and magnesium in blood serum have proved difficult for routine diagnostic purposes. The ammonium oxalate–ammonium phosphate method tended to give difficulty with the precipitates. The end point of the complexometric method varied with operators and even from day to day with any single operator.

Pribil & Roubal (1954) reported that polarographic estimation of calcium in serum and urine was possible with a recording polarograph. This would be of value in routine estimations as it would replace much of the human element with an electronic device. Their method is based on the polarographic determination of Zn^{2+} ions quantitatively displaced from an ammoniacal zinc–ethylenediaminetetra-acetic acid complex by Ca^{2+} ions present in biological material. The authors stated that although a similar quantitative displacement of Zn^{2+} ions by Mg^{2+} ions does not occur, there is some displacement and consequently the presence of magnesium affects the result of the calcium determination. They therefore added saturated ammonium phosphate solution to precipitate magnesium before estimating the calcium.

We studied their method but have not been able to confirm its value for the determination of calcium in blood sera. We did not, however, use the same type of polarograph. We used one in which the pen-recording device is replaced with a cathode-ray tube (K 1000, Southern Instruments, Crawley, England). This gives much greater sensitivity and records the rate of change of current and not the actual diffusion current. This rate of change is greatest at the half-wave potential, a constant for any given element in any particular electrolyte. Maximum suppressors are rarely necessary and, apart from serum itself, were not used in this work. One advantage of such instruments is that the entire polarogram is reproduced every 7 sec., as the whole record is made during the life of a single mercury drop. Furthermore, since the instrument is very sensitive it is possible to restrict the amount of material for analysis.

The differences between the two types of instruments should not have affected the results, but we found that under our conditions magnesium in serum was not precipitated quantitatively by ammonium phosphate. If the magnesium was less than 3 m-equiv./l., then little or no magnesium was precipitated by the ammonium phosphate. When, however, this amount was exceeded, partial precipitation occurred. It was with such high concentrations of magnesium that Pribil & Roubal were working. Their method will, however, yield approximately correct results for calcium in normal sera since a depressive effect in the peak current due to the presence of serum is offset by the additive effect of the magnesium. This would not be true for serum in cases of hypocalcaemia.

We were able, however, to elaborate a method which we find simple, rapid and accurate.

MATERIALS

Glass-distilled water was used in the preparation of all solutions.

Ammoniacal zinc–ethylenediaminetetra-acetic acid. This consisted of 6·6 mM-disodium ethylenediaminetetra-acetate (EDTA) and 6·6 mM-zinc sulphate dissolved in aqueous 8N-ammonia solution. This was the concentration used by Pribil & Roubal (1954) in their work. As they state, a zinc wave is obtained even in the blank and must be subtracted from subsequent readings. It varies between 2·0 and 2·5 μA according to the solution used, as very slight inaccuracies in the amount of either zinc sulphate or EDTA will affect the reading.

Stock 0·01 M-*calcium chloride.* Calcium carbonate (A.R. grade) was dried at 110° and dissolved in the minimum amount of hydrochloric acid. Carbon dioxide was removed by boiling and the solution diluted to the required volume.

Stock 0·01 M-*magnesium chloride.* This was prepared from magnesium oxide ignited and cooled in a desiccator and dissolved in the minimum amount of hydrochloric acid and diluted to the required volume.

Blank serum. Since proteins of the type present in serum tend to decrease the diffusion current, it is necessary to include serum in the standard solutions. Ox and sheep sera were therefore passed through an ion-exchange column (25 cm. × 1 cm.^{2}) consisting of the sodium form of Amberlite resin IR-120 in order to remove all calcium and magnesium.

RESULTS

In the preparation of their standard solutions, Pribil & Roubal (1954) added measured amounts of calcium chloride to the ammoniacal zinc–EDTA reagent and included saturated ammonium phos-

phate. We found that the addition of ammonium phosphate lowered the peak-current reading. A similar effect was given by serum. This depressive effect is shown in Fig. 1. The same effect is shown when zinc sulphate is added to the ammoniacal zinc–EDTA reagent in place of calcium.

We therefore prepared a series of standard solutions of calcium and magnesium with treated serum added. These solutions are detailed in Table 1, which also gives the peak-current readings. These show that calcium and magnesium react as a single substance present to the amount of the combined total. The curve constructed from the figures in the second and third columns is therefore a standard curve for total calcium and magnesium in the test solution.

A similar standard curve was prepared for magnesium alone. To do this, measured amounts of magnesium chloride were added to a solution containing treated serum and calcium. A constant volume of ammonium oxalate was added to precipitate the calcium and to produce conditions similar to those in the unknown sera to be tested. The details are given in Table 2, and show that again the peak-current readings bear a linear relationship to the amount of magnesium added.

In order to determine whether or not these peak-current readings represent a stoicheiometric displacement of Zn^{2+} ions from the ammoniacal zinc–EDTA reagent, they were compared with the readings obtained by substituting equivalent amounts of zinc. Fig. 2 shows that, in solutions without serum, equivalent amounts of zinc and of calcium and magnesium produce the same peak current. However, when treated serum is included in the solutions the results for Ca^{2+} and Mg^{2+} ions are not identical with those for Zn^{2+} ions.

Fig. 1. Depression of peak-current readings by serum and ammonium phosphate. ●, 1 mM-Calcium chloride added to 12·5 ml. of ammoniacal zinc–EDTA reagent and diluted to 25 ml. with water; ○, 1 ml. of saturated ammonium phosphate added; ▲, 2·5 ml. of serum (from which calcium and magnesium had been removed) added; △, 1 ml. of saturated ammonium phosphate and 2·5 ml. of treated serum added. The value of the current produced by the supporting electrolyte in each case has been subtracted.

Application of the results to the assay of blood sera

To 5 ml. of ammoniacal zinc–EDTA reagent in a stoppered cylinder, 1 ml. of serum and 4 ml. of water were added and the mixture was well shaken.

Table 1. *Standard solutions of calcium and magnesium*

Solutions containing various amounts of 1 mM-calcium chloride and either 0·5 mM- or 1 mM-magnesium chloride were added to 12·5 ml. of ammoniacal zinc–EDTA reagent and 2·5 ml. of treated serum to give the ratios in the first column. The total volume was adjusted to 25 ml. by adding water. Amounts shown in the second column give the concentrations in m-equiv./l. of calcium and magnesium in the solutions assayed. A drop of octanol was added to the solution in the polarograph cell to prevent frothing.

Ratio Ca : Mg	Total Ca + Mg	Peak current (μA)
2 : 1	0·12	1·30
1 : 1	0·16	1·70
2 : 3	0·20	2·10
4 : 1	0·20	2·10
2 : 1	0·24	2·50
4 : 3	0·28	2·95
1 : 1	0·32	3·40
2 : 3	0·40	4·15
4 : 1	0·40	4·15
2 : 1	0·48	5·35
4 : 3	0·56	6·00
6 : 1	0·56	6·00
3 : 1	0·64	7·00
2 : 1	0·72	7·75
8 : 1	0·72	7·75
4 : 1	0·80	8·75
8 : 3	0·88	9·50

Table 2. *Standard solutions of magnesium*

Ammonium oxalate solution (4 %; 0·5 ml.) and 0·25 ml. of 1 mM-calcium chloride solution were added to the volumes given in columns 1, 2 and 3 to give a final volume of 2·5 ml. in each case. These solutions were prepared in centrifuge tubes and allowed to stand for at least 4 hr. before centrifuging at 3000 rev./min. The supernatants were poured into cylinders containing 5 ml. of ammoniacal zinc–EDTA reagent and 2·5 ml. of water. After shaking, a portion was placed in the cells, octanol was added, and the peak current determined. Figures in column 4 give the concentration of magnesium in the solution assayed.

Vol. of treated serum (ml.)	Vol. of water (ml.)	Vol. of $MgCl_2$ (ml.)	Mg (m-equiv./l.)	Peak current (μA)
1·0	0·25	0·50 (0·5 mM)	0·05	0·46
1·0	0·50	0·25 (2 mM)	0·10	0·96
1·0	0·25	0·50 (2 mM)	0·20	1·96
1·0	0	0·75 (2 mM)	0·30	2·76
0·75	0	1·00 (2 mM)	0·40	3·81

A drop of octanol was added and the peak current determined. From this was subtracted the reading of a blank solution (5 ml. of zinc–EDTA reagent, 1 ml. of treated serum and 4 ml. of water) and the total calcium and magnesium in the serum was then obtained from the standard curve.

To obtain the amount of magnesium, 1 ml. of serum was added to 1 ml. of water in a centrifuge tube, followed by 0·5 ml. of 4% ammonium oxalate. The mixture was allowed to stand for at least 4 hr. and, after centrifuging for 10 min. at 3000 rev./min., the supernatant was poured into 5 ml. of ammoniacal zinc–EDTA reagent with 2·5 ml. of water and a drop of octanol. The solution was shaken and the peak current determined. After subtraction of the value of the current produced by the blank (1 ml. of treated serum and 2·5 μmoles of calcium chloride substituted for the unknown serum) the concentration of magnesium in the serum was obtained from the standard graph. The amount of calcium can then readily be obtained by difference.

The accuracy of the method was checked by the estimation of 20 replicates of one ox and of one sheep serum. Each of these replicates was prepared from a single serum, starting afresh for each. At the same time a chemical estimation was made by the method of Clark & Collip (1925). The figures given in Table 3 compare the reproducibility of the two methods.

In addition the method was applied to the routine estimation of calcium and magnesium in sera. Fifty individual samples, both of ox and sheep sera, were estimated in duplicate by polarographic and chemical methods. Table 4 gives the results obtained.

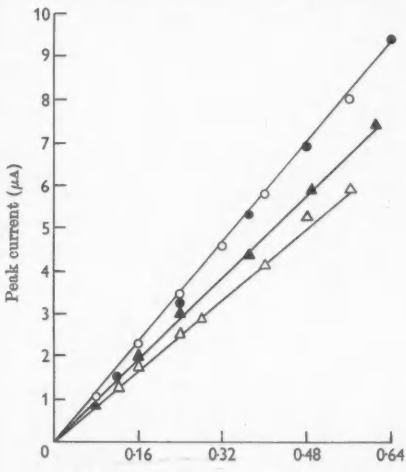

Fig. 2. Effect of serum on the relationship between Zn^{2+} ions added and Zn^{2+} ions displaced by added Ca^{2+} and Mg^{2+} ions. ●, Various amounts of 1 mM-zinc sulphate were added to 12·5 ml. of ammoniacal zinc–EDTA reagent and the volume was adjusted to 25 ml. with water; ○, equivalent amounts of calcium chloride and magnesium chloride (ratio varied) in place of zinc sulphate; ▲, zinc sulphate together with 2·5 ml. of treated serum; △, calcium and magnesium chloride together with 2·5 ml. of treated serum.

DISCUSSION

Tables 3 and 4 show that the accuracy and reproducibility of the polarographic method compare favourably with those of the precipitation method. The main difficulty with this latter method is a compromise between inadequate washing of the precipitate and possible loss of the precipitate during the removal of excess of oxalate and phosphate. This does not occur in the polarographic method and the estimations of calcium in the

Table 3. *Duplicate polarographic and chemical analyses of 20 replicates each of one ox and one sheep serum*

Results are given in m-equiv./l. and refer to means ±S.D.

Serum	Method of estimation	Mg	Ca
Ox	Polarographic	1·6±0·06	4·7±0·11
	Chemical	1·6±0·13	4·6±0·09
Sheep	Polarographic	2·0±0·12	5·1±0·17
	Chemical	2·0±0·06	5·2±0·12

Table 4. *Comparison of polarographic and chemical analyses of 50 individual samples of ox and of sheep sera*

The mean differences were obtained by subtracting the results of the chemical analyses from those obtained by the polarographic method, with subsequent division of the sum of the differences by the total number of sera. The standard deviations of these means were calculated and probabilities P obtained from a table for distribution of t.

		Range (m-equiv./l.)	Mean difference $\bar{x}$	S.D. of mean $S_{\bar{x}}$	t $(\bar{x}/S_{\bar{x}})$	P
Ox	Mg	1·6–3·3	0·01	0·033	0·423	$0·6 < P < 0·7$
	Ca	2·75–6·65	0·07	0·039	1·794	$0·05 < P < 0·1$
Sheep	Mg	1·4–3·4	0·05	0·030	1·666	$0·1 < P < 0·2$
	Ca	2·95–8·6	0·03	0·036	0·833	$0·4 < P < 0·5$

different samples of sheep and ox sera tended to be slightly higher than those obtained from the permanganate titration.

The concentration of calcium and magnesium varied little in the majority of samples tested, therefore the differences shown by the two methods are unlikely to depend on the relative concentrations of calcium and magnesium. In the greater proportion of samples these differences, whether positive or negative, were within 5% of the total amounts of calcium or magnesium. A few, however, showed differences up to a maximum of 0·85 m-equiv./l. for calcium and 0·6 m-equiv./l. for magnesium. Table 4 shows that the difference between the two methods is not statistically significant.

The displacement of zinc from its complex with EDTA by calcium is possible in the presence of the high concentrations of ammonia which were used (Schwarzenbach, 1957). This was demonstrated experimentally by Pribil & Roubal (1954). The relation between added calcium and magnesium and zinc displaced is stoicheiometric, since the same peak current is obtained whether equivalent amounts of calcium and magnesium or zinc are added (Fig. 2). Since we obtained a linear relationship between calcium plus magnesium added and the peak-current readings (Table 1), whatever the proportions of calcium and magnesium, it is apparent that at the concentrations we used, both calcium and magnesium displace zinc from the complex to an equal extent. In the preparation of the standard magnesium graph, however, the figures obtained for magnesium alone are slightly lower than those for equivalent amounts of calcium plus magnesium. This is due to incomplete decantation of the comparatively viscous supernatant, since similar graphs prepared without serum were identical.

Certain buffers, including phosphate, hinder the displacement of Zn^{2+} ions from ammoniacal zinc–EDTA by calcium or magnesium. Thus ammonium phosphate would be expected to depress the peak current of zinc displaced by calcium from zinc–EDTA. This is so, but when zinc itself is added to the solutions instead of calcium, an equal depression occurs at all concentrations investigated. Thus the depression of the current cannot be due to any effect of PO_4^{3-} ions on the dissociation of zinc–EDTA by calcium since, if it were, the curve for added zinc itself would not be equally depressed. Nor is it due to the formation of complex zinc phosphate ions, since the half-wave potential is the same whether ammonium phosphate is present or not. A new equilibrium must be set up with some slight precipitation of zinc phosphate. Despite the depressive effect of added ammonium phosphate, however, the relationship between zinc and calcium remains stoicheiometric, confirming this observation of Pribil & Roubal.

When serum is present, however, the relation between calcium plus magnesium added and zinc displaced ceases to be stoicheiometric: the curves diverge as the concentration increases (Fig. 2). In addition, certain complex zinc ions are formed, since the half-wave potential is raised from $-0{\cdot}126$ to $-0{\cdot}12$.

In view of the complexity of serum, similar solutions to those in Table 1 and Fig. 2 were prepared with 5% gelatin (passed through an ion-exchange column) instead of serum. Unlike serum, gelatin depressed the peak current by a constant amount, regardless of the concentration of zinc sulphate added, and had no effect on the half-wave potential of the solutions. Nevertheless, the presence of gelatin did affect the displacement of zinc by calcium and magnesium. Hence the effect of serum is, first, the protein effect of lowering the peak current for any given concentration of zinc and the effect of partial inhibition of the displacement of zinc by calcium and magnesium; secondly, a more specific effect of serum is to cause greater inhibition of the peak current in zinc solutions as the concentration rises.

The importance of incorporating serum into the standard solution is therefore obvious.

SUMMARY

1. A polarographic method for the estimation of calcium and magnesium in sera is described in which the total calcium and magnesium is determined and then the magnesium alone. The calcium concentration is found by difference. This method is as accurate as, and faster than, available chemical procedures.

2. The method was checked by the determination of 20 replicates each of one sheep and one ox serum. It was then applied to the analysis of 100 individual sera (50 ox and 50 sheep). None of the results differed significantly from those obtained by chemical methods, though the polarographic estimates of calcium tended to be slightly higher.

3. The presence of serum in the supporting electrolyte affects the stoicheiometry of the reaction. This effect is more complex than that of the simple protein gelatin.

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